Proteotoxicity links therapy-induced cancer cell senescence to Alzheimer's disease

Dissertation
zur Erlangung des akademischen Grades

Doctor rerum naturalium (Dr. rer. nat.)

im Promotionsfach Biologie

eingereicht an der Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin

von

Dhriti Dhawan, M.Sc.

Präsidentin der Humboldt-Universität zu Berlin
Prof. Dr.-Ing. Dr. Sabine Kunst

Dekan der Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin
Prof. Dr. Bernhard Grimm

Gutachter

1. **Prof. Dr. med. Clemens A. Schmitt**
2. **Prof. Achim Leutz**
3. **Prof. Thomas Sommer**

Tag der mündlichen Prüfung: 23rd May, 2018

“It always seems impossible until it’s done”
- Nelson Mandela

CONTENTS

ABBREVRIATIONS

AD	Alzheimer's disease
ADR	Adriamycin
AICD	APP intracellular domain
APH-1	Anterior pharynx-defective 1
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
Aβ	Amyloid beta
BrdU	5-bromo-2'-deoxyuridine
Calm	Calmodulin
Casp	Caspase
CDK	Cyclin-dependent kinase
CHK	Checkpoint homologs
CTF	C-terminal fragment
CTX	Cyclophosphamide
DDR	DNA damage response
DNA	Deoxyribonucleic acid
Ece	Endothelin converting enzyme
EOAD	Early onset Alzheimer's disease
ER	Endoplasmic Reticulum
FAD	Familial Alzheimer's disease
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSEA	Gene set enrichment analysis
HDAC	Histone deacetylase
IF	Immunofluorescence
IHC	Immunohistochemistry
IL6	Interleukin 6
IL8	Interleukin 8
KEGG	Kyoto Encyclopedia of Genes and Genomes
LOAD	Late-onset Alzheimer's disease

LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LRP	Lipoprotein-receptor related protein
MAP1-LC3	Microtubule-associated protein light chain3
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
NCSTN	Nicastrin
NF- κB	Nuclear factor- κB
OIS	Oncogene-induced senescence
PCR	Polymerase chain reaction
PEN2/PSENEN	Presenilin enhancer 2
pFTAA	Pentameric- formyl thiophene acetic acid
PI	Propidium iodide
PSEN	Presenilin
RB	Retinoblastoma
ROS	Reactive oxygen species
RQ-PCR	Taqman® based real time PCR
SA-β-gal	Senescence-associated β-galactosidase
SAHF	Senescence-associated heterochromatic foci
SASP	Senescence associated secretary phenotype
shRNA	Small hairpin RNAs
Sort	Sortilin
TGF	Transforming growth factor
TIS	Therapy-induced senescence
UPR	Unfolded protein response
UT	Untreated

SUMMARY (English)

A senescent cell experiences proteotoxic stress in consequence to senescence-associated secretory phenotype (SASP). SASP factors involve increased production of cytokines and chemokines, which overload and disrupt protein homeostasis. This induces endoplasmic reticulum (ER) stress, unfolded protein response (UPR) as well as elevated autophagic-lysosomal burden in senescent cells, further promoting the formation of misfolded proteins.

In this thesis we found, in Eμ-*myc* transgenic mice lymphomas, therapy-induced senescence (TIS) by Adriamycin or Cyclophosphamide treatment leads to an up regulation of genes involved in Alzheimer's disease (AD). However, this is true only when TIS correlates with SASP, leading to proteotoxicity. On the other hand, we demonstrate that transgenic AD inflicted mice also present with features of senescence. Mutations of genes such as amyloid precursor protein (*APP*) and presenilin1 (*PSEN1*), not only engender AD, but also augment senescence. In *APPPS1*$^{+/-}$ transgenic mice, the senescent cells co-occur with amyloid β (Aβ) plaques. Likewise, in neuroblastoma cell line SH-SY5Y, *APP* overexpression results in upregulation of senescence markers, including markers for ER stress and autophagic-lysosomal burden. These observations imply that there is a crosstalk between senescence and AD.

We demonstrate proteotoxicity as a common denominator between the two pathologies. However, we found that *APP* and *PSEN1* are not regulators of TIS. APP contributes to proteotoxic stress associated with TIS as knocking down of APP leads to a reduction of ER stress. Inhibiting Aβ formation by targeting the processing of APP using Semagacestat- a gamma-secretase inhibitor, selectively targets senescent cells resulting in cell death. This indicates its senolytic potential. However, Semagacestat has no impact on the expression of ER stress markers. This suggested that in Eμ-*myc* lymphomas, APP contributes to ER stress in an Aβ-independent fashion, unlike in AD, where Aβ is considered central to its pathophysiology.

SUMMARY (German)

Eine seneszente Zelle erfährt proteotoxischen Stress durch einen Prozess, der als “Seneszenz-assoziierter sekretorischer Phänotyp (SASP)” bezeichnet wird. SASP Faktoren involvieren vermehrte Produktion von Cytokinen und Chemokinen, die die Proteinhomöostase stören. Das führt zu Stress im Endoplasmatischen Retikulum (ER), Unfolded Protein Response (UPR) sowie erhöhter Autophagie in seneszenten Zellen. In dieser Arbeit zeigen wir, dass Therapie-induzierte Seneszenz (TIS), ausgelöst durch Adriamycin (ADR) oder Cyclophosphamid (CTX), zu einer erhöhten Expression von Alzheimer-assoziierten Genen führt. Das ist allerdings nur der Fall, wenn TIS mit SASP und Proteotoxizität korreliert. Außerdem zeigen wir, dass transgene Mäuse mit Alzheimer ebenso Eigenschaften von Seneszenz aufweisen. Mutationen in Genen wie Amyloid-Precursor-Protein (APP) und Presenilin 1 (PSEN1) erzeugen nicht nur Alzheimer, sondern verstärken auch Seneszenz. In APPPS1$^{+/-}$ transgenen Mäusen treten seneszente Zellen zusammen mit Amyloid β (Aβ) Plaques auf. Gleichfalls führt die Überexpression von APP in der Neuroblastom Zelllinie SH-SY5Y zu einer Erhöhung von Seneszenz-Markern, einschließlich ER Stress und Autophagie-assoziierten Lysosomen. Diese Beobachtungen implizieren einen Zusammenhang zwischen Seneszenz und Alzheimer.

Wir präsentieren Proteotoxizität als gemeinsamen Nenner dieser beiden Pathologien. Allerdings kommen wir zu dem Ergebnis, dass APP und PSEN1 keine Regulatoren von TIS sind. APP trägt zur TIS-assoziierten Proteotoxizität bei, da ein knock-down von APP zu einer Reduzierung des ER Stress führt. Außerdem, können seneszente Zellen mittels Blockierung der APP Spaltung und somit Bildung von Aβ Plaques durch den Gamma-Sekretase Inhibitor Semagacestat, selektiv getötet werden. Das deutet auf ein senolytisches Potential hin. Allerdings hat Semagacestat keinen Einfluss auf die Expression von ER Stress Marker. Dies suggeriert, dass APP in Eμ-*myc* Lymphomen Aβ-unabhängig zu ER Stress beiträgt, während diese in Alzheimer eine zentrale Rolle in der Phathophysiologie spielen.

INTRODUCTION

1 Cellular senescence

Cellular senescence is a phenomenon that limits the propagation of a normal cell and leads it to a state of growth arrest[1]. Unlike quiescence, this cell cycle arrest is stable as the cells fail to re-enter the mitotic cell cycle, even when the stress inducing senescence is removed[2]. Senescence is an alternative mechanism to restrict potentially harmful cells from proliferating. In general, senescence can be triggered in a normal cell by various cellular stresses, such as telomere uncapping and DNA damage response (DDR). Particularly, cancer cells can enter senescence via activation of an oncogene (oncogene-induced senescence/OIS) or radio- and chemotherapy that generally induces DDR (therapy-induced senescence/TIS)[2–6]. Other senescence inducing stimuli include reactive oxygen species (ROS), and unfolded protein response elements[7–9].

Key features of a senescent cell are G1 phase arrest of the cell cycle, adoption of a typical flat and vacuole rich cytoplasmic morphology, while continuing their cellular metabolic activities. Senescent cells are synonymous with an enhanced lysosomal activity of the β-galactosidase enzyme. This is used as a tool for detection of senescent cells, using X-gal as a substrate[10].

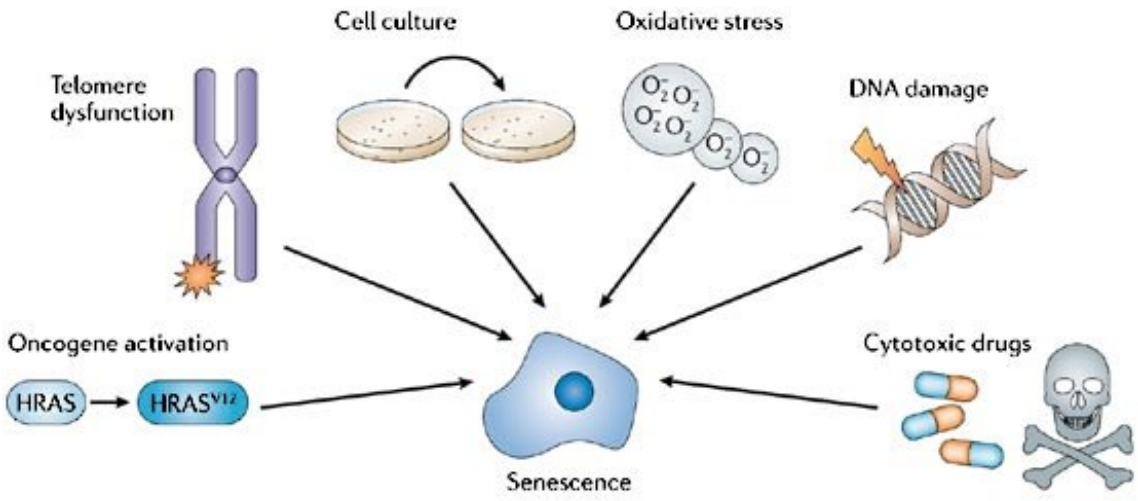


Figure 1: Various modes of senescence induction. These stimuli are usually present in the tumor environment. Induction of senescence acts as a protective mechanism to prevent the proliferation of cancerous cells. Figure from Collado et al. 2006[5].

1.1 Oncogene-Induced Senescence

OIS acts as a cellular defense mechanism that is initiated upon activation of an oncogene. Approximately 50 oncogenes possess the ability to initiate an OIS response. Ras was first reported to induce senescence when overexpressed in human fibroblasts[6,11]. Many downstream pathways of Ras are involved in terminal cell cycle arrest, for e.g. the MAPK cascade and PI3 kinase/Akt pathway. The Ras pathway is involved in the promotion of translocation and activation of FoxO transcription factors, depending on the signaling events[12]. Alternatively, loss of tumor suppressors such as PTEN, can also initiate an OIS response[13]. Therefore, genes that initiate uninhibited proliferation could potentially lead to senescence.

The role of OIS has been established *in vivo,* where it deters tumor formation at an early stage[14–17]. For instance, Ras driven mouse lung adenoma/adenocarcinoma display a presence of cells under cell cycle arrest, mimicking features of cellular senescence[17]. However, inactivation of senescence by genomic deletion of Suv39h1 in B-cell lymphomas permits these cells to evade the tumor-suppressive potential associated with the oncogenic Ras induced senescence[16]. Malignancy is also observed in case of loss of tumor suppressor PTEN, despite the induction of cellular senescence. In this scenario, the activated oncogene is unable to induce senescence because loss of PTEN leads to inactivation of p53, which in turn bypasses senescence[18]. Thereby suggesting that OIS does not always succeed to form a barrier between uncontrolled cell proliferation and tumor formation.

Apart from oncogenes that induce OIS, such as Ras and Raf, some other oncogenes do not have the same response. For instance they induce apoptosis as their primary cellular failsafe response, such as via c-Myc[6,19–21].

1.2 Therapy-Induced Senescence (DNA-Damage Response)

In cases where cancer cells evade the tumor suppressing OIS, the cells become immortal and proliferate in an uncontrolled manner[22]. While this suggests that possibly the senescence machinery is faulty in these cancer cells, these cells remain responsive to TIS. This could be because there are various different pathways that are involved in senescence regulation[23–25]. There also remains a probability that

cells are required to retain some levels of cell cycle regulators to ensure mitotic cell divisions[26]. Hence, when OIS fails to act as a failsafe mechanism, TIS can be used as an alternative method to curb tumor formation and growth.

Senescence can also be induced by an increase in the intracellular levels of ROS. This is true for mortal as well as immortal cells, by causing DNA, lipid or protein damage[27]. For instance, oxidative stress induced by Zn^{2+} ionophore pyrithione is known to induce growth arrest leading to senescence[28,29]. Moreover, PTEN hemizygous cells undergo TIS when treated with PTEN inhibitor VO-OHpic. This was induced in cancer cells in mouse models as well as human xenografts[13]. This leads to a conclusion that susceptibility of some cancers to TIS can be targeted via some stress-signaling responses. It was also observed that in these cases, TIS occurred independent of DDR[13].

1.3 The Eμ -*myc* mouse lymphoma model

The Eμ-*myc* mouse lymphoma model was derived from a mouse plasmocytoma. The transgenic mouse with a rearranged *c-myc* oncogene was generated[30]. The Eμ-*myc* transgene constitutes an insertion of 361 bp 5' to the *c-myc* gene on chromosome 15, which consists of a 2.3 kb segment of the immunoglobulin heavy (H) chain locus from chromosome 12. This insertion includes the lymphoid- specific enhancer from the Ig heavy chain locus region and interferes with the normal expression of c-*myc*. The resulting Eμ-*myc* transgenic mice express the transgene predominantly in B-lymphoid cells and engender the development of B cell lymphomas which resemble human non-Hodgkin lymphomas[31]. This model is beneficial since the resulting lymphomas can be detected by palpation of the peripheral lymph nodes and can hence be subjected to treatment in vivo or ex vivo. Lymphoma cells can be cultured for a limited period *in vitro*, allowing genetic modifications (e.g. by retroviral infection) and treatments. When apoptosis is blocked by overexpression of bcl2, for example, these cells homogeneously enter senescence and serve an excellent model system for TIS[32].

1.4 Molecular pathways of senescence

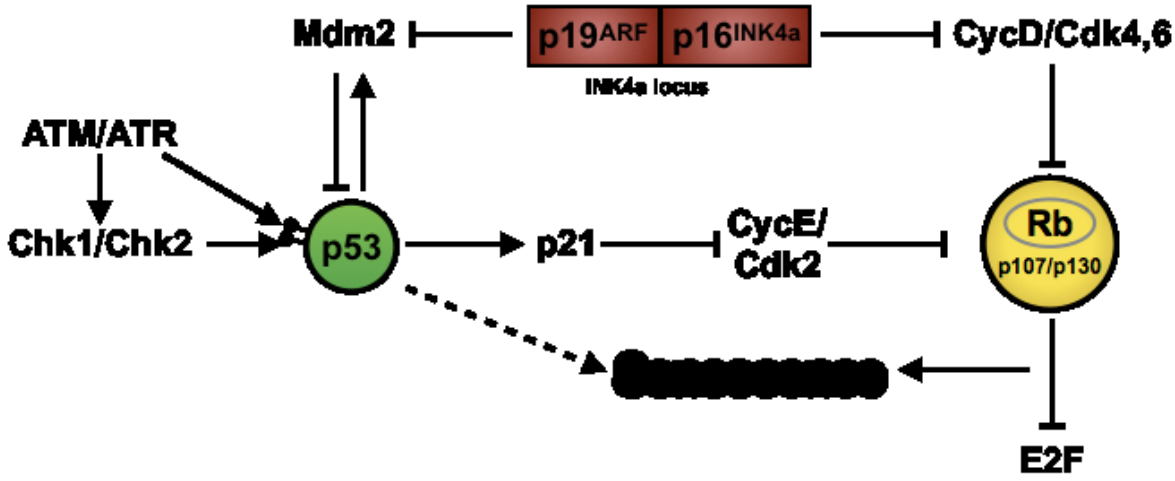


Figure 2: p53 and Rb - the key regulators of senescence. Rb is activated by p53 via p21 as well as other factors. Rb inhibits transcription of E2F target genes, thereby activating senescence. Via p21 and p16INK4a are two pathways of Rb activation. Phosphorylation of p53 is essential for senescence execution. Players such as ATM/ATR and Chk1/Chk2 proteins, p19ARF- product of the INK4a locus responsible for impeding mdm2 are all factors contributing to the activation of p53. Figure modified from Porath and Weinberg 2005[4].

DDR is induced as a result of hindered DNA replication machinery and/or reactive oxygen species (ROS)[33–36]. The DDR induces a kinase dependent activation of the tumor suppressor p53, via the ataxia telangiectasia–mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinase pathways. The intensity of these responses and the specific underlying pathways vary between cell types[13]. Stable activation of p53 protein further activates its targets such as cyclin-dependent kinase (CDK) inhibitors p16, p15, p21 and p27[23]. CDK-cyclin inhibition results in proliferative arrest, whereas senescence is induced via the hypo-phosphorylated form of Rb[37]. Activation of p21 via p53 therefore serves as a relevant marker for senescence[38,39].

Both p53 and Rb are necessary for the senescence initiation[4]. Inactivation of p53 in mouse embryonic fibroblasts (MEFs) prevents the cells from undergoing senescence as well as allows re-entry into the cell cycle[40,41]. Therefore, p53 and Retinoblastoma (Rb) are key to cell cycle regulation, since they regulate most of the players involved in cell cycle progression and play an essential role in senescence.

Rb is one of the most important regulators of the G1 to S transition of the cell cycle. The active form of Rb, bound to the E2F family, leads to the repression of their

transcriptional targets[42], which in turn induces senescence. E2F transcription factors regulate genes that promote entry into the S phase of the cell cycle[43]. Rb activity is regulated via the p53/p21pathway, or via the $p16^{INK4a}$ pathway, or in parallel by both[4]. Hence, a combination of p53 and Rb pathways determines the final level of stress experienced by the cell. This decides the ultimate cellular response that is activated[4]. Rb and $p16^{INK4a}$ are essentially the cell cycle players associated with maintaining the irreversibility of senescent cells back into the cell cycle[6,44–48].

The $p16^{INK4a}$ protein is a commonly used marker for senescence. Its expression could be induced by different stresses as well as by senescence[49–51]. It indirectly regulates the activation of Rb by inhibiting Cyclin D/Cdk4 complex that would otherwise phosphorylate and inactivate Rb[43,52]. Inactivation of Rb releases E2Fs to initiate the corresponding transcriptional program. Mouse Eμ-myc transgenic lymphomas with Bcl2 overexpression undergo senescence in response to chemotherapy in a $p16^{INK4a}$ dependent manner[53].

Furthermore, there are several epigenetic modifications associated with senescence. Trimethylation of Lys9 on histone H3 (H3K9me3) modifications and heterochromatin protein 1 (HP1) binding, especially at E2F target genes, are universally used to indicate the senescent population in cell culture. Rb along with HP1 and histone methyltransferases associate themselves with E2F promoters during senescence, possibly forming senescence-associated heterochromatic foci (SAHF) and establishing senescence[42,45]. However, SAHFs have limitations serving as a marker for senescence since they appear in human but not always in mouse senescent cells[54–56].

Since many players are involved in various aspects of senescence, but not all of them might be expressed in all cell types. Taken together in combinations, they can be used as molecular markers of senescence.

1.5 Senescence-Associated Secretory Phenotype

Senescence is also associated with secondary features such as Senescence Associated Secretory Phenotype (SASP)[57]. SASP is a complex phenotype, where senescent cells increase the expression of secreted proteins, including enzymes responsible for the degradation of extracellular matrix as well as NF-κB dependent pro-inflammatory cytokines[58]. It includes pro-inflammatory cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8), chemokines like monocyte chemoattractant proteins (MCPs) and macrophage inflammatory proteins (MIPs), immune modulators, growth factors such as transforming growth factor-β (TGFβ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and proteases[57,59,60]. SASP is arguably observed to have contravening roles of promoting senescence and in some cases promoting tumorigenesis[61–66] (Figure 3). These factors could cause inflammation and could be essential for the phagocytotic clearance of senescent cells[63,67]. Hence, SASP can constitute a microenvironment that could possibly lead to the removal of senescent cells, through its intrinsic paracrine and autocrine activities[23]. On the other hand, SASP is also noted for its ability to trigger senescence, in a paracrine manner, via the ROS and DNA-damage pathways [62,68,69]. Thus, SASP factors contribute towards many biological processes which may or may not be adept to clear harmful cells.

SASP contributes to the proteotoxic stress associated with senescence, by exhausting the capacity of cells to maintain accurate protein synthesis, post-translational processing, vesicular transport and secretion. Thus, autophagy is also induced in these conditions, which could be an effort to cope with this proteotoxic stress[70].

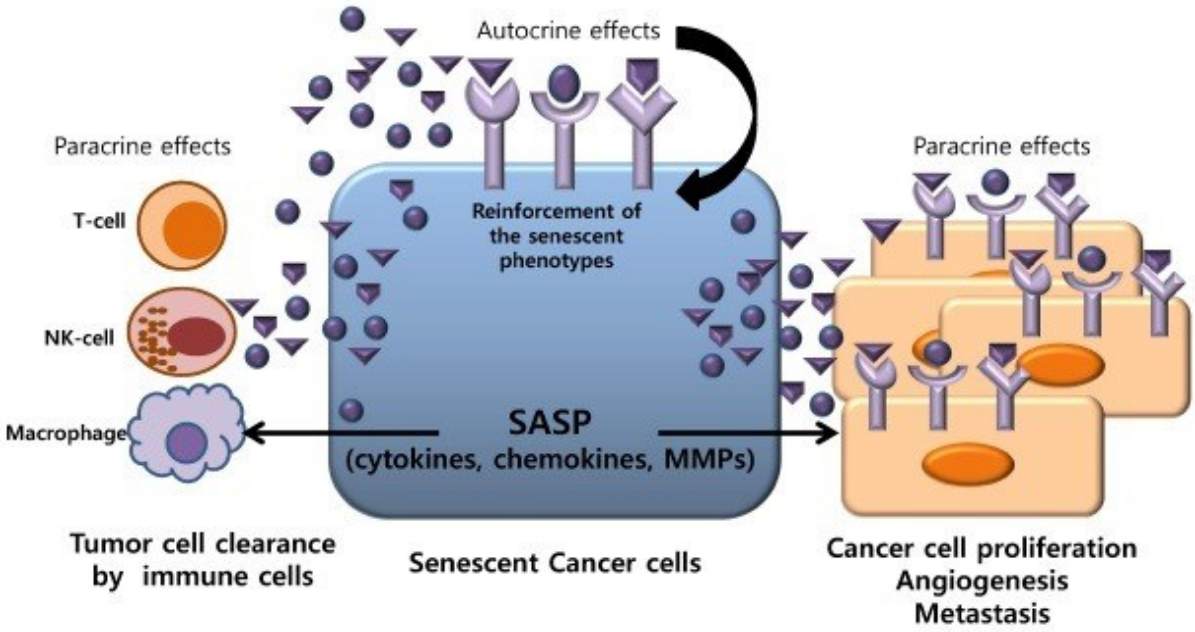


Figure 3: Senescence-associated secretory phenotype (SASP). SASP involves secretion of factors contributing to the maintenance of the senescent state and growth arrest of senescent cells (the autocrine effect), as well as influence the tissue microenvironment (the paracrine effect). The autocrine effect comprises production of proinflammatory factors such as cytokines and chemokines, and matrix-remodelling factors that are responsible for alteration of the tissue microenvironment enhance cancer cell proliferation, angiogenesis, as well as metastasis. There are anti-inflammatory factors that are released by the senescent cells which encourage the elimination of tumor cells by marking them for immunoclearance. On the other hand, as a paracrine effect, some factors exert tumor promoting activity on nearby cancer cells by increasing tumorigenesis. Figure adapted from Lee et al. 2014[71].

Recently, SASP has been compared with an evolutionarily conserved mechanism that governs cellular competition and bi-directional paracrine signaling amongst weaker and stronger cells[72]. This could be an effort to maintain tissue homeostasis. OIS cells appear to be surrounded by immune cells expressing $p16^{INK4a}$ and $p21^{CIP1}$, observed in *in vivo* models. These surrounding cells make it difficult to propose a senescence associated marker expression in tissues. It also complicates the interpretation of how the OIS cells *in vivo* affect the surrounding stromal environment and immune response. Besides, most of the known SASP components have many others functions apart from senescence, for e.g. in acute bacterial or viral infections, inflammation, wound healing and growing malignancies[73]. IL-6, which is considered as one of the signature SASP components have a much more pronounced expression induced by some acute inflammation as compared to in senescence[74,75]. Currently, a signature to distinguish between senescence and other forms of cellular stresses characterized by NF-κB is unavailable[73].

1.6 Proteotoxicity in senescence

To sustain harmony between the components of the complex proteostatic network, a cell maintains proper protein homeostasis or proteostasis. An imbalance in any branch of the complex network can have dire consequences. In general, it could lead to the breakdown of the proteostatic network and contribute towards numerous metabolic, cardiovascular, oncological and neurodegenerative diseases. Therefore, it is important to understand the proteostatic network regarding different biological contexts and the implication of proteostasis across various diseases.

The cell experiencing proteotoxicity withstands the stress via proper autophagic activity in the cytosol or the ER, since large amounts of proteins are synthesized there[76]. An imbalance in these autophagic and ER systems results in an incompetency to clear the misfolded or unstable proteins inside the cells. The accumulation of such proteins leads to cytotoxic effects[77]. In aging cells, a decline in coordination of the proteostatic network has been shown to increase the burden of misfolded proteins without efficient protein synthesis, processing, localization and degradation pathways[78,79] Apart from aging, cellular senescence (TIS and OIS) with massive SASP requires increased protein production, and thereby promotes the chance of misfolded protein formation. Dörr and colleagues established that this protein production is responsible for TIS associated higher glucose utilization and ATP production. They also linked this observation to SASP, since models lacking a strong SASP response did not present with these features. Moreover, only SASP producing TIS cells exhibited ER-stress-UPR-ubiquitination autophagy cascade. These features together are representative of proteotoxicity[70].

Mammalian target of rapamycin complex 1 (mTORC1) regulates autophagy. mTORC1 inactivates ATG13 and ULK1 by phosphorylating them, depending on the nutrition levels[80]. Starvation or decreased glucose and high AMP levels lead to autophagy induction, via AMPK and repression of the mTORC1 activity. Whereas, increased levels of amino acids, insulin and insulin-growth factor 1 inhibit autophagy via the PI3K/AKT/mTORC1 pathway induction[81]. In senescent primary human fibroblast, there is a significant upregulation of the autophagosomal marker LC3-II[82]. In different cellular contexts, mTOR inhibition has a different impact[83]. In OIS, SASP is affected by recruiting the mTOR complex to a novel sub-cellular compartment

called the TOR-autophagy spatial coupling compartment (TASCC)[84]. Inhibiting mTOR via rapamycin interferes with autophagy and induces senescence in radioresistant cancer cells in a xenograft model, but suppresses SASP[85,86]. Thus, autophagy modulates senescence and its associated cellular mechanisms. Although there is a considerable role of autophagy in senescence in cancer, not much is known about their co-dependence on each other.

2 Alzheimer's Disease

Alzheimer's disease (AD) is one of the primary causes of dementia in the world. AD can also be referred to as a protein misfolding disease due to the formation of abnormally folded beta amyloid (Aβ) and tau amyloid proteins in the brain. Characteristic features of AD include neuron loss, neuronal dystrophy, senile plaques, neurofibrillary tangles, and loss of synapses. Amyloid precursor protein (APP) is divided into smaller fragments of Aβ by enzymes such as β-secretase and γ-secretase. Accumulation of these Aβ peptides triggers neuron degeneration[87]. The underlying mechanism responsible for such pathophysiology remains to be resolved. Although there is evidence which supports that plaques formed by the accumulation of Aβ are central to the AD pathology, it does not support all the aspects of AD. There is encouraging indication that Aβ or amyloid independent factors add to the pathogenesis of the disease, such as alternative AD-related genes (like Apolipoproteins E and microtubule-associated protein tau), elevated autophagic burden, inflammation, and oxidative stress[88]. This suggests that there may be more than one biological process that can lead to AD.

AD is classified depending on the age of onset of the disease. Early onset AD (EOAD) (also known as Familial AD or FAD) occurs before the age of 65 years and is observed in approximately 5% of all cases. On the other hand, late-onset AD (LOAD) occurs at 65 years or later, and is observed in more than 95% of the affected cases. There is a difference between the pathology of the two types of ADs. EOAD results in a more rapidly progressing AD and follows a Mendelian pattern of inheritance. APP along with Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2), which are components of the γ-secretase enzyme, are known to be involved in Aβ generation, which is the central component involved in the pathophysiology of EOAD. In more than 85% cases of EOAD, a mutation is observed in these three genes. Therefore, these three are useful as diagnostic biomarkers of EOAD. On the contrary, LOAD follows a non-Mendelian pattern. It is observed that the probability to develop LOAD is twice if a first-degree relative has LOAD[87].

Although the physiological function of APP is largely unknown, it is proposed to be involved in trafficking, neurotrophic signaling, cell adhesion and cell signaling[89–91]. PSEN1, which is a critical component of the γ-secretase complex, is known to play

an important role in autophagic vacuole clearance[92]. Induction of autophagy in mouse fibroblasts by rapamycin or nutrient deprivation result in translocation of the γ-secretase complex from the endosomal/ER pool to the autophagic vacuoles and the γ-secretase activity in these vacuoles increase the production of Aβ[93]. Therefore, increased residence time of these autophagic vacuoles in cells increases the chance of Aβ production and exocytosis. In AD mouse models, over expressing the FAD-related mutant human PSEN1 and APP, macroautophagy leads to the accumulation of Aβ in the autophagic vacuoles of the affected neurons[94,95]. In APP/PSEN1 transgenic mice (harboring Thy1- APPKM670/671NL and Thy1-PS1L166P mutations), autophagic vacuoles appear in neurons at a younger stage before Aβ plaques appear. This suggests that macroautophagy is an early response and is followed by amyloid deposits and not the other way around[93].

Autophagy is also an integral part of senescence and possibly these AD inducing genes could also be involved, directly or indirectly, in senescence.

2.1 APP processing

APP can be proteolytically processed via two alternative pathways- the amyloidogenic and the non-amyloidogenic pathway[96,97](Figure 4). In amyloidogenic pathway, APP is cleaved by the enzyme BACE (β-site APP cleaving enzyme) at the β-secretase site[98]. This results in a soluble β-APP fragment (sAPPβ) and the C99 fragment, which is a 99-amino acid C-terminal fragment (CTF). C99 is attached to the membrane but can be subsequently cleaved by a γ-secretase complex[99]. This process takes place in the intramembrane region and releases the Aβ peptide. Aβ peptides can further aggregate extracellularly to form plaques. On the other hand- via the non-amyloidogenic pathway, APP is first cleaved by a α-secretase that cleaves within the Aβ region, and releases a soluble 110-120 kDa α-cleaved APP fragment (sAPPα)[100]. Hence, the resulting CTF, with 83-amino acids, is shorter and known as C83. C83 can be further cleaved by γ-secretase to release p3. In both the pathways, cleavage of their respective CTF by γ-secretase also releases APP intracellular domain fragment (AICD) [90,101]. These various components formed via APP processing may contribute to both disease and normal physiology.

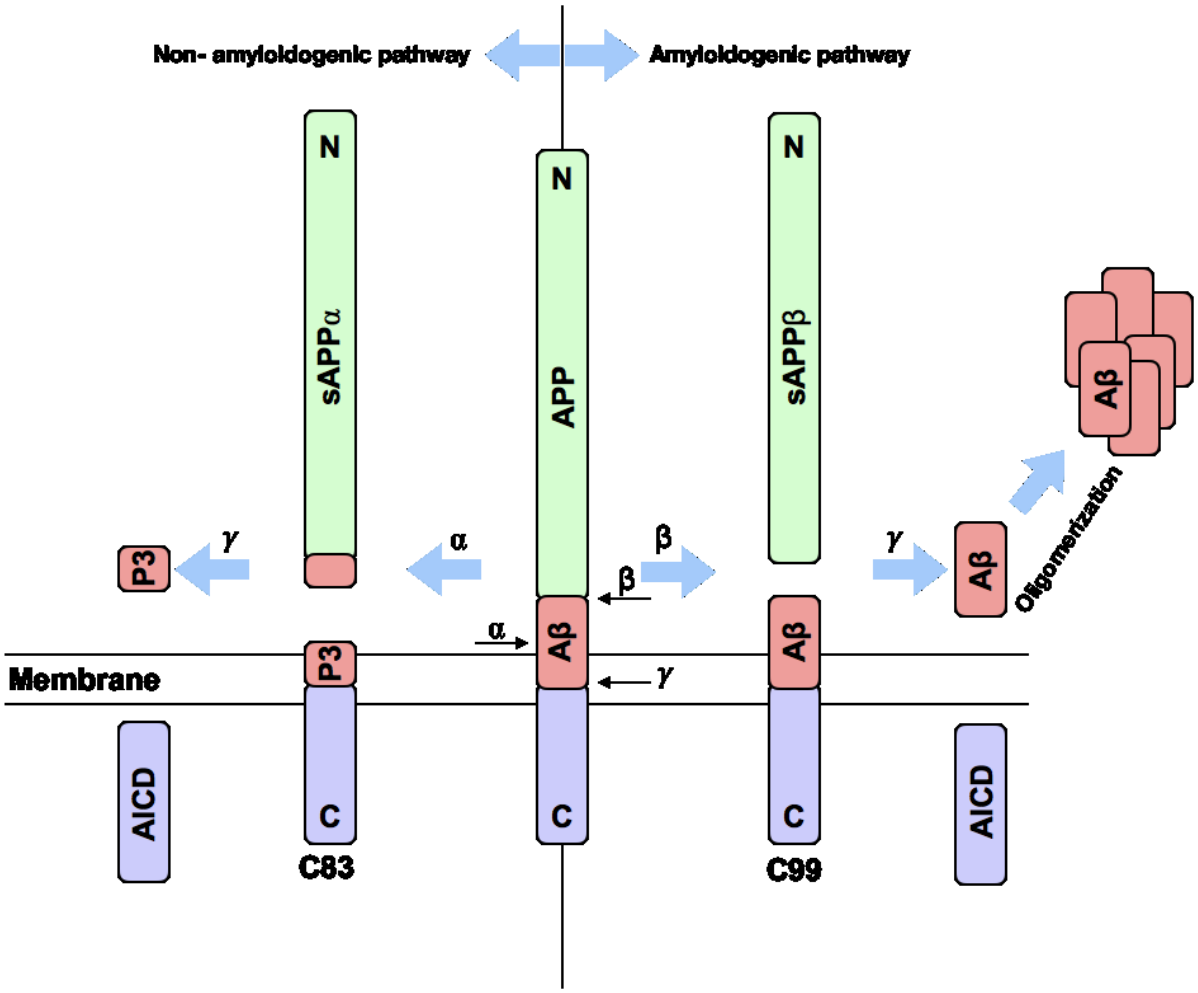


Figure 4: Proteolytic processing of APP. Two major pathways of APP processing- the amyloidogenic and the non-amyloidogenic pathway. β-secretase cleaves APP resulting in the subunits sAPPβ and C99 in case of the amyloidogenic pathway, and α-secretase cleaves APP resulting in the subunits sAPPα and C83 in case of the non-amyloidogenic pathway. In both the pathways, the γ-secretase complex cleaves the APP-CTF into AICD and Aβ (from C99) and p3 (from C83) peptides. Figure adapted from Nicolas et al. 2014.

2.2 Gamma-secretase complex

γ-secretase, an aspartyl protease, is a complex enzyme with over 90 substrates[102]. It is composed of 4 subunits forming a 1:1:1:1 heterodimer, which is PSEN, nicastrin (NCSTN), anterior pharynx-defective 1 (APH-1) and presenilin enhancer 2 (PEN2)[103–107]. PSEN is the catalytic subunit for γ-secretase[108–110]. In mammals, there are two isoforms, PSEN1 and PSEN2. PSEN mutations lead to either a change in the $A\beta_{1-42}$/ $A\beta_{1-40}$ peptides ratio or an increase in the overall Aβ levels[111,112]. These features are associated with EOAD[113]. But it is still unclear whether the loss of or gain of function of PSEN1 mutation is involved in AD[114,115].

γ-secretase is a tightly regulated enzyme and only a small fraction of its complexes are active at a time[116–118]. All the subunits are present in the active complexes, and overexpression of all the subunits is necessary for increasing γ-secretase activity[119]. However, *in vivo*, overexpressing PSEN alone leads to increased enzyme activity[120]. This suggests that regulation of γ-secretase is more complex *in vivo* and probably has many layers of functional regulations in the context of specific tissues or diseases [121].

Before the discovery of γ-secretase, some proteasome inhibitors were tested in APP-transfected cells where they resulted in the accumulation of APP-CTFs and inhibited the production of p3 and Aβ[122–124]. Initial studies of a γ-secretase inhibitor, DAPT, reported that γ-secretase inhibition resulted in potential inhibition of Aβ production in cells. Much higher oral doses of the inhibitors, as compared to *in vitro* studies, were required to observe 50% decline in the Aβ levels in the brains of young APP transgenic mice[125]. Subcutaneous application of the drug affected the Aβ levels in plasma and CSF, decreasing it in a dose-dependent manner. But this only resulted in reduced Aβ levels in the brains of the younger mice and not in the older mice[126]. None of the tested inhibitors succeeded in suppressing Aβ plaques formed in the brains of the adult animal models.

Semagacestat is a γ-secretase inhibitor that advanced into phase III clinical trials. This compound resulted in a reduction in the steady-state levels of Aβ in the plasma, but not in the cerebrospinal fluid[127–129]. In the phase III of the trial, drug administration led to severe gastrointestinal toxicity, immunomodulation, and skin

cancer. These effects are indicative of an impaired Notch activity. Another intriguing finding was that the cognition of drug-treated group was worse than the placebo-treated group in AD patients. Therefore, apart from its nonselective effect on Notch signaling, this suggests a potential negative impact of lowering brain Aβ levels, or more so of increasing the APP-CTF levels[130]. Thus, despite of being a lucrative target for AD treatment, γ-secretase inhibition has not succeeded as a viable approach due to its undesirable off-target effects. An approach that specifically and exclusively targets APP processing needs to be further elucidated.

3 Senescence and Alzheimer's disease

Senescent cells are a component of age-related pathologies, such as neurodegenerative diseases and malignancies[131]. This suggests that these pathologies might be driven by features associated with senescent cells[132]. Protein synthesis is one such process that is regulated by checking for misfolded proteins. The cellular proteome clears these misfolded proteins by correcting or degrading them. Age impairs cellular activity, which leads to misfolded proteins[133]. The accumulation of oxidative modifications such as carbonylation, oxidized methionine, and glycation, along with an increase of cross-linked and aggregated proteins in aged tissues suggest that these features significantly contribute to their functional decline. Aged tissues also associate with less catalytically active enzyme populations[134].

According to the senescence hypothesis of Alzheimer's disease progression, the AD associated factors induce stress to the neurons, which in turn influence their function and survival. Once the stress inducing factors surmount the cell's threshold to endure the given stress, senescence pathways are triggered. There is crosstalk between the pathways involved in senescence and the APP proteolytic system. Senescence-associated signaling has also been shown to regulate APP proteolysis[135].

In APP overexpressing EOAD, there is a potential imbalance between functions of PSEN, depending on its activity in the γ-secretase complex. An increase in demand for PSEN activity in one system leads to low availability of PSEN for its roles in other proteolytic systems such as Notch, LRP and CD44. This mimics some of the changes to PSEN function (associated with the γ-secretase complex) according to the PSEN hypothesis[136–138]. The hypothesis states that mix of gain and loss of functions of PSEN1 and to some extent PSEN2, dependent or independent of its γ-secretase functions, contribute to the progression of AD[114]. There are mutations of the PSEN gene that lead to an increased affinity of γ-secretase towards APP. This results in elevated production of Aβ in general, or modification of the ratio of fragments $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ due to altered cleavage, causing Aβ to accumulate and form plaques[113,139]. However, the clear role of Aβ in affecting the neuropathology and cognitive status of an AD patient is not known[140]. An

interpretation of this observation could be the loss of alpha pathway cleavage within the APP proteolytic system. That is, if the mutations result in loss of PSEN function, it could affect the balance between alpha and beta pathways, which could lead to loss of protective function. Alternately, the ratio between different Aβ fragments could be responsible for different signaling functions[114,141–143]. This suggests that there could be many different causes and contributors of the AD biology, which results in a complicated AD pathology.

Notch has myriads of functions and is also involved in cancer, where it functions like a tumor suppressor when affiliated with p53 activation. Together, Notch and p53 suppress proliferation by their role in regulating differentiation, senescence or apoptosis[144]. APP has been proposed to be involved in regulation of notch processing because of cross-talk in between them[145–147]. Hence, APP could have an impact on progression of the disease due to altered Notch signaling, and be involved in determining the fate of the cells by regulating senescence and cell death[148,149].

Spontaneously occurring LOAD could be a result of progressing cellular senescence in the brain, and this could be due to accumulation of ageing features leading to the pathology. The factors associated with LOAD are mostly involved in cell signaling pathways responsible for neuronal survival and act to induce stress in the cell. Senescence pathways are triggered once these cells cannot negate the effects of stress anymore. When the neuroprotective mechanisms are unable to cope with stresses associated with the disease, senescence progresses[135].

It has been postulated that once these cells undergo senescence, Aβ is released as a protective response, to induce ROS activation and induce factors such as IL-1 and IL-6 from the neighboring senescent cells. Aβ can hence be used as a marker for senescence in this scenario. As the senescence in the neurons progresses, there is an inevitable loss of neural network function and failure of cognitive processes. Alternatively, in the case of EOAD, the cells undergo premature senescence due to the stress induced by the imbalance between APP/PSEN. This imbalance nudges the cell towards senescence. The disease hence progresses with senescence. In familial forms of AD, the mechanisms of neuroprotection might be exhausted earlier and the stress is elevated[135].

Senescence also strengthens the association between cancer and neurodegenerative pathways[150–155]. Therefore, it is possible that senescence may have the possibility to lay the groundwork for AD progression[156–158]. This might be pivotal to use the knowledge from one discipline for the benefit of the other.

HYPOTHESIS

Preliminary data from our lab demonstrated that AD genes were up regulated in case of TIS of Eμ-*myc*-driven B-cell lymphomas with bcl2 overexpression. According to the senescence hypothesis of AD, factors associated with Alzheimer's can trigger senescence[135]. Proteotoxic stress has been reported in both AD and senescence[70,159]. This led us to hypothesize that proteotoxicity might be the common denominator of the two pathologies and players of the Alzheimer's pathology could be the contributing factor of senescence-related proteotoxicity.

AIM

To explore the link between cellular senescence and Alzheimer's disease (AD), and to provide novel insights into both pathologies, using Eμ-*myc*-driven B-cell lymphomas. This may lead to re-purposing of therapeutic strategies across distinct disease entities to move cancer research forward.

MATERIALS AND METHODS

1 MATERIALS

1.1 Chemicals

Name	Company
30% Acrylamide/Bis-acrylamide	Carl Roth
Adriamycin	Sigma
Agar	Carl Roth
Agarose	Carl Roth
Albumin Fraktion V	Carl Roth
Ammoniumperoxisulfat (APS)	Carl Roth
Ampicilin, sodium salt	Carl Roth
Blasticidin	Invitrogen
Bromphenol blue	Euro-bio
Calcein	Sigma
Calcium chloride ($CaCl_2$)	Carl Roth
Chloroform	Merck
Chloroquine-diphosphate	Sigma
DAPI	Sigma
Diethyl pyrocarbonate (DEPC)	Sigma
Dimethylsulfoxid (DMSO)	Merck
dNTPs	Biochrom
Dulbecco´s MEM	Invitrogen
DTT	Eurobio
ECL reagent	Millipore
Ethylenediaminetetraacetate (EDTA)	Carl Roth
Ethanol, absolute	Carl Roth
Ethidium-bromide powder	Sigma
Fetal bovine serum (FBS)	Biochrom
Formaldehyde solution 37%	Carl Roth
Gel Ruler 1-kb ladder plus	Fermentas

Glucose	Carl Roth
Glutaraldehyde	Carl Roth
Glycerol	Carl Roth
Glycine	Serva
H_2O_2	Invitrogen
Hydrocloric acid, HCl	Sigma
HEPES	Carl Roth
Hygromycin	Invitrogen
Iscove's modified Eagle's media	Invitrogen
L-Glutamine (solution)	Invitrogen
L-Glutamine (powder)	Biochrom
Magnesium chlorid [MgCl2x6H2O]	Carl Roth
β-Mercaptoethanol	Carl Roth
Methanol	Carl Roth
Microscopy Aquatex	Merck
Milk powder	Carl Roth
Mowiol® 4-88	Merck
Nonident 40 (NP-40)	Fluka
PageRuler Plus Prestained Protein Ladder	Thermo Scientific
Paraformaldehyde	Sigma
Penicillin-streptomycin	Biochrom
Pentameric formyl thiophene acetic acid (PFTAA)	Gift from Prof. Dr. Frank Heppner
Phosphate Buffered Saline (PBS)	Carl Roth
Phenylmethylsulfonylfluoride (PMSF)	Sigma
Polybrene	Sigma
Potassium bicarbonate [KHCO3]	Sigma
Potassium ferricyanide [K3Fe(CN)6]	Sigma
Potassium ferrocyanide [K4Fe(CN)6x3H2O]	Sigma
Potassiumchloride (KCl)	Sigma
Potassiumacetate (KAc)	Carl Roth
ProLong™ Diamond Antifade Mountant	Thermo Scientific
Propidium iodide	Sigma

Protease inhibitors tablet	Roche
Puromycin	Calbiochem
ROTI-prestained	Invitrogen
RotiQuant, Bradford reagent	Carl Roth
RPMI 1640	Invitrogen
Salmon sperm DNA	Invitrogen
SDS 2326.2 ROTH	Carl Roth
Sodiumchloride (NaCl)	Carl Roth
Sodium citrat [$C_6H_5Na_3O_7x2H_2O$]	Carl Roth
Sodium-desoxycholate	Sigma
Sodiumfluoride (NaF)	Sigma
Sodiumhydroxide (NaOH)	Carl Roth
Sodiumorthovanadate	Sigma
Sodium pyrophosphate [$Na_4P_2O_7x10H_2O$]	Sigma
Tris(hydroxymethyl) base	Carl Roth
TEMED	Sigma
Triton-X 100	Merck
TRIZOL	Invitrogen
Trypan-blue solution	Sigma
Trypsin-EDTA	Carl Roth
Trypton/Pepton	Carl Roth
Tween 20	Carl Roth
Vecta-Shield, mounting medium	Vector
X-Gal	Carl Roth
Xylene-cyanol	Eurobio
Yeast extract	Carl Roth
2-propanol	Sigma
5-bromo-2'-deoxyuridine (BrdU)	Sigma

1.2 Equipments

Name	**Company**
Avanti J-26XP Ultra-Speed centrifuge	Beckman Coulter
Bacteria shaker Innova 4000	New Brunswick Scientific

Bioanalyzer 2100	Agilent Technologies
Cell culture dishes and falcons	TPP
Cell culture incubator	Heraeus
Centrifuge 5417R	Eppendorf
Centrifuge Rotina 35R	Hettich
ChemiDoc MP Imaging System	Bio-Rad
Chemocam imager	Intas
Cryotubes	Sefar company
Cyto chambers	Hettich
FACS Calibur	Becton Dickinson
Fluorescence microscope	Zeiss
Fluorescence microscope BZ-9000	Keyence
gentleMACS™	Miltenyi
Guava® easyCyte flow cytometer	Merck
LUNA™ Automated Cell Counter	Logos biosystems
Megafuge 1.0 R	Eppendorf
Microscope Zeiss Telaval 31	Zeiss
Microscopy Immersion Oil	Merck
Microwave	Siemens
Mr. Frosty Freezing Container	Thermo Scientific
MSD® MULTI-SPOT Assay system	Meso Scale Diagnostics
NanoDrop ND-2000	Peqlab
Nylon Mesh (35 µm)	Simport Plastics
PCR machine	Peqlab
pH-meter MP-220	Mettler Toledo
Pipettes	Eppendorf
Polystyrene tubes	BD Falcon
PVDF membrane (Immobilon-P)	Millipore
Rotilabo Filter sterile (0.22 µM PVDF)	Carl Roth
Rotilabo Filter sterile (0.45 µM PVDF)	Carl Roth
Scalpel for single-use	Feather
SDS-PAGE Chamber	C.S.B. Scientific
Semi-dry transfer system	Biorad

Serological pipettes Falcon	Becton Dickinson
StepOnePlus ™ Real-Time PCR System	Applied Biosystems
Thermo-cycler PCR machine	Peqlab
Thermomixer	Peqlab
UV detection system	Biometra T13
Whatman paper (3MM)	Schleicher-Schuell

1.3 Antibodies

Name	Clone Number	Company
Actin	4967	Cell signaling
Anti-β-amyloid (Purified)	4G8	Biolegend
Anti-mouse IgG HRP	NXA931	Amersham
Anti-mouse-IgG AlexaFluor 488	A11001	Thermofisher Scientific
Anti-mouse-IgG AlexaFluor 594	A11005	Thermofisher Scientific
Anti-rabbit IgG HRP	NA9340	Amersham
Anti-rabbit-IgG AlexaFluor 488	A11008	Thermofisher Scientific
Anti-rabbit-IgG AlexaFluor 594	A11037	Thermofisher Scientific
APP	2452	Cell signaling
APP 22C11	MAB348	Millipore
APP C-termial (751-770)	171610	Millipore
APP-C terminal	A8717	Sigma
H3K9me3	ab8898	abcam
Ki67	SP6	Thermofisher Scientific
LAMP2a	Ab18528	Abcam
LC3	2G6	Nanotools
p16 (F-12)	Sc-1661	Santa cruz
p21	sc-397	Santa Cruz
p53 phospho-S15	9284	Cell signaling
p53 Protein (CM5)	NCL-p53-CM5p	novocastra

Psen1	Ab53717	abcam
Psen1 (APS18)	AB15458	abcam
Ras	sc-68743	Santa Cruz Biotechnology
p62(p62/SQSTM1)	5114	Cell signaling
Tubulin	T6199	Sigma
V-ATPase A1 (E-8)	sc-374475	Santa cruz

1.4 Enzymes

Name	**Company**
BamH1	NEB
Bgl II	NEB
EcoR I	NEB
Hind III	NEB
Proteinase K	Merck
RNAse A	Fluka
RNAse-out RNAse Inhibitor	Invitrogen
Superscript Reverse Transcriptase III	Invitrogen

1.5 Commercial Kits

Name	Catalog Number	Company
Dako REALTM Detection System, Alkaline Phosphatase_RED, Rabbit_Mouse	K5005	Dako
Guava ViaCount Reagent for Flow Cytometry	4000-0040	Millipore
Immobilon Western Chemiluminescent AP Substrate	P36600	Millipore
MSD® MULTI-SPOT Assay System Aβ Peptide Panel 1 (4G8) Kit	K15199G-1	Meso Scale Diagnostics
NucleoSpin Gel and PCR Clean-up	740609.250	MACHEREY-NAGEL
ON-TARGETplus Mouse App siRNA: SMARTpool	L-043246-00-0005	GE Healthcare Dharmacon
ON-TARGETplus Mouse Psen1 siRNA: SMARTpool	L-048761-01-0005	GE Healthcare Dharmacon
ON-TARGETplus Non-targeting Pool	D-001810-10-05	GE Healthcare Dharmacon
QIAprep Spin Miniprep Kit	27104	Qiagen
SuperScript® III First-Strand Synthesis System	18080-051	Invitrogen
TaqMan® Gene Expression Master Mix	4369016	Applied Biosystems

1.6 Mouse strains

Name	Reference
C57BL/6	Clarence Cook Little, Bussey Institute, Harvard University, USA, 1921
C57BL/6 (Suv39h1 knock-out)	Peters *et al*, Cell 2001, IMP, Vienna, Austria
C57BL/6 (p53 knock-out)	Jacks *et al*, Current Biology 1994, Whitehead institute, Cambridge, USA
APPPS1$^{+/-}$	Gift from Prof. Dr. Frank Heppner

1.7 Cell strains and cell lines

Name	Description	Source
NIH 3T3	mouse embryonic fibroblast	ATCC
Pheonix	human embryonic kidney line	Clontech/Takara
SH-SY5Y	Human neuroblastoma cell line Parental and overexpressing APP	Gift from Prof. Dr. Thomas Willnow
Eμ-myc B cell lymphoma	Murine primary lymphoma	Lab made
MEF	Murine embryonic fibroblast	Lab made

1.8 Bacteria

DH5α E.coli	**Genotype:** F´ proA+B+ laclq Δ lacZ M15/ fhuA2 Δ(lac-proAB) glnV gal R(zgb-210::Tn10)TetS endA1 thi-1 Δ(hsdS-mcrB)5

1.9 Plasmids

Name	Backbone
MSCV-Bcl2-GFP	MSCV-GFP
MSCV-Bcl2-puro	MSCV-puro
pSR-shAPP-puro	pSuper-retro-puro
pSR-shPsen1-puro	pSuper-retro-puro
MSCV-IκBΔN-GFP	MSCV-GFP
MSCV-Ras-puro	MSCV-puro

1.10 Primers

Name	Sequences
Short hairpin APP	**Forward:** GATCCCC CGTTACAGTAGCACTCCTAAT TTCAAGAGA ATTAGGAGTGCTACTGTAACG TTTTTA **Reverse:** AGCTTAAAAA CGTTACAGTAGCACTCCTAAT TCTCTTGAA ATTAGGAGTGCTACTGTAACG GGG
Short hairpin Psen1	**Forward:** GATCCCC CCCAAAGTTTACTCAAGACTA TTCAAGAGA TAGTCTTGAGTAAACTTTGGGTTTTTA **Reverse:** AGCTTAAAAACCCAAAGTTTACTCAAGACTA TCTCTTGAA TAGTCTTGAGTAAACTTTGGG GGG
Real-Time PCR primers	
Human APP	Taqman primer from Applied Biosystems Cat. Hs00169098_m1
Human Psen1	Taqman primer from Applied Biosystems Cat. Hs00997789_m1
Mouse Adam10	Taqman primer from Applied Biosystems Cat. Mm00545742_m1
Mouse Aph1a	Taqman primer from Applied Biosystems Cat. Mm00778687_s1
Mouse Apoe	Taqman primer from Applied Biosystems Cat. Mm01307193_g1
Mouse APP	Taqman primer from Applied Biosystems Cat. Mm01344172_m1
Mouse Calm1	Taqman primer from Applied Biosystems Cat. Mm01336281_g1

Mouse Casp3	Taqman primer from Applied Biosystems Cat. Mm01195085_m1
Mouse Casp7	Taqman primer from Applied Biosystems Cat. Mm00432322_m1
Mouse Ece1	Taqman primer from Applied Biosystems Cat. Mm01187104_m1
Mouse Gsk3b	Taqman primer from Applied Biosystems Cat. Mm00444911_m1
Mouse Htt	Taqman primer from Applied Biosystems Cat. Mm01213820_m1
Mouse Ide	Taqman primer from Applied Biosystems Cat. Mm00473077_m1
Mouse IL23a	Taqman primer from Applied Biosystems Cat. Mm00518984_m1
Mouse IL6	Taqman primer from Applied Biosystems Cat. Mm00446190_m1
Mouse LPL	Taqman primer from Applied Biosystems Cat. Mm00434764_m1
Mouse LRP1	Taqman primer from Applied Biosystems Cat. Mm00464608_m1
Mouse Ncstn	Taqman primer from Applied Biosystems Cat. Mm01293323_g1
Mouse Psen1	Taqman primer from Applied Biosystems Cat. Mm00501184_m1
Mouse Psen2	Taqman primer from Applied Biosystems Cat. Mm00448406_m1
Mouse Psenen	Taqman primer from Applied Biosystems Cat. Mm00727761_s1
Mouse Psmb8	Taqman primer from Applied Biosystems Cat. Mm00440207_m1
Mouse Sort1	Taqman primer from Applied Biosystems Cat. Mm00490905_m1

1.11 Media and Solutions

1.11.1 Media

Name	Ingredients
LB Medium	10 g tryptone 5 g yeast extract 10 g NaCl 1 L dH2O, pH 7.0 store at +4°C
LB agar plate	LB-medium+15 g Agar After autoclaving, cool to 36pprox.. 55°C, add antibiotic and pour into petridish store at +4°C
B Cell medium	DMEM + IMDM (1:1) + 10% FCS + Penicillin-streptomycin (100 U/ml) + 4 mM L-Glutamine + 25 µM β-mercaptoethanol store at +4°C
DMEM medium	DMEM + 10% FCS + Penicillin-streptomycin (100 U/ml) store at +4°C
Freezing medium	FCS + 10 % DMSO store at +4°C

1.11.2 Solutions and Buffers:

Transfection solutions	
2 M $CaCl_2$	2.99 g in 10 mL dH_2O, store at -20°C
2x HBS (Hepes buffered saline)	280 mM NaCl 10 mM KCl 1.5 mM Na_2HPO_4 x $2H_2O$ 12 mM Dextrose 50 mM HEPES 100 mL dH_2O, pH 7.05
100 mM Chloroquine	0.516 g Chloroquine diphosphate 10 ml dH_2O store at -20°C
Western blotting solutions	
Protein lysis buffer (NP-40)	1% NP-40 50 mM Tris-HCl pH 7.4 150 mM NaCl 5 mM $MgCl_2$ proteinase inhibitor cocktail (Roche) phosphatase inhibitor cocktail (Roche) store at -20°C
SDS sample buffer	1 ml 0.5 M Tris-HCl (pH 6.8) 0.8 ml Glycerol 1.6 ml 10% SDS 0.4 ml 14.3 M β-mercaptoethanol 0.4 ml of 1% Bromephenol blue store at -20°C
0.5 M Tris-HCl pH 6.8	6 g Tris base, pH 6.8, upto100 ml dH_2O
1.5 M Tris-HCl, pH 8.8	27.23 g Tris base, pH 8.8, upto 100 ml dH_2O
10% APS	1 g Ammonium peroxysulfate in 10 ml dH_2O

10x Running buffer (pH 8.3)	30 g Tris base 144 g Glycine 10 g SDS upto 1 L dH_2O pH 6.8
Transfer Buffer	2.9 g Tris base 14.5 g glycine 200 ml methanol upto 1 L dH_2O, store at 4°C
25x TBS	60 g Tris base 200 g NaCl 9.5 ml 10 N HCl upto 1 L dH_2O
1x TBS-Tween (TBS-T)	0.2 % Tween 20 in TBS
Blocking solution	5 % dry milk in 1x TBS-T, freshly prepared
SA-β-gal assay solutions	
PBS / $MgCl_2$	1 mM $MgCl_2$ in 1 x PBS pH 5.5 for murine sample pH 6.0 for human sample
Fixation solution	0.25 % Glutaraldehyde 2% Paraformaldehyde in PBS, freshly prepared
20x Potassium cyanide (KC) stock	20 mg $K_3Fe(CN)_6$ 1.050 mg $K_4Fe(CN)_6 \times 3H_2O$ in 25 ml 1 x PBS store at 4°C in the dark
40x X-Gal solution	40 mg X-Gal in 1 ml DMFO Store at -20°C
Staining solution	9.25 ml PBS / $MgCl_2$ 0.5 ml 20 x KC solution 0.25 ml 40 x X-Gal solution freshly prepared

Immunofluorescent (IF)/ Immunohistochemistry (IHC) staining	
Fixation solution	4% Paraformaldehyde in 1 x PBS freshly prepared
Permeabilization solution	0.2%Triton X-100 in 1 x PBS
Blocking solution	1% BSA in 1 x PBS
Washing buffer	0.01% Tween 20 in 1x PBS

2 METHODS

2.1 Molecular Cloning of DNA construct

2.1.1 PCR: amplification of DNA

PCR primers were designed using online tools and used for their respective DNA targets. The total volume of PCR reactions was 50 µl.

Components	Quantity
Template DNA	10- 100 ng
10x PCR buffer with $MgCl_2$	5µl
dNTP mix (10 mM each)	1µl
Sense primer (10 µM)	1µl (10 pM)
Antisense primer (10 µM)	1µl (10 pM)
Taq or Pfu Polymerase (5 U/µl)	1µl
dH_2O	Add dH_2O upto 50 µl.

Conditions	Temperature	Duration
Initial denaturation	94°C	
30-35 cycles	94°C	30 sec
	48-60°C	30 sec
	72°C	1 minute/kb DNA
Final extension	72°C	10 min
Hold	4°C	∞

2.1.2 PCR product purification and digestion

NucleoSpin PCR clean-up kit was used to clean up the PCR products as per the manufacturer's protocol. This was followed by digestion of the product using the respective restriction enzymes for a minimum of 6 hours, at 37 °C.

2.2 Ligation of DNA fragments

Purified linearized vector (~50 ng) and PCR product were used in a ratio of 1:3. T4 ligase was used for the ligation reactions (10 µl), which were carried out according to the manufacturer's instructions.

2.2.1 Bacteria transformation

Ligation product (10 µl) was added to 50 µl competent DH5α E.coli, in a 1.5 ml eppendorf tube. This mix was incubated on ice for 20 minutes. The cells were heat shocked at 42°C for exactly 90s and immediately put on ice for 2 minutes. After adding 1 ml LB medium, the tube was incubated in 37°C shaker for 45 minutes. The bacteria was then pelleted and plated on LB-agar plates with 100 µg/ml ampicillin. The plates were incubated at 37°C overnight.

2.2.2 Plasmid preparation: mini-preparation and maxi-preparation

Individual colonies were picked and incubated in individual tubes with 3 ml LB medium (100 µg/ml ampicillin) at 37°C overnight. Bacteria was centrifuged at 3000 rpm (5 minutes, 4°C) and pellet was resuspended, lysed, precipitated by sequentially adding 100 µl ice-cold Solution I, 200 µl freshly prepared Solution, 150 µl of ice-cold Solution III. After centrifugation (14000 rpm, 10 minutes, 4°C), supernatant was transferred to a new Eppendorf tube and 1 ml of ethanol was added to precipitate DNA. After vortexing and centrifugation (14000 rpm, 10 minutes, 4°C) the pellet was washed with 1 ml of 70% ethanol. DNA was air-dried and dissolved in 30 µl dH2O.
Maxi-preparation of plasmid was made using the Invitrogen maxi-prep kit, following the manufacturer's protocol.

2.2.3 DNA sequencing

The DNA sequencing was delegated to Source BioScience LifeSciences (MDC, Berlin).

2.3 Cell culture

2.3.1 Thawing and freezing of cells

Thawing: Cryovials with frozen cells were removed from liquid nitrogen or -80°C, and placed into a 37°C water bath. The thawed cells were transferred to 9 ml fresh growth medium and were pelleted by centrifugation at 1,200 rpm for 5 min. All these steps were carried out swiftly, to minimize the toxic effects of DMSO on the cells. The cell pellet was resuspended, seeded on tissue culture dishes and cultivated in the incubator.

Freezing: Adherant cells were trypsinized, whereas, for suspension cells, the cells were collected from the culture dish and transferred to the falcon tubes for centrifugation, pelleted down and resuspended in freezing medium and transferred into sterile 1.5 or 2 ml cryovials. The cells were then put into “Mr. Frosty” box and immediately transferred to -80°C freezer. For long-term storage, cells were stored in liquid nitrogen.

2.3.2 Adherent cell culture

Cancer cell lines, NIH 3T3 and Phoenix cells were cultivated at 37°C in an incubator with 5% CO2, 20% O2, 95% humidity atmosphere. Murine embryonic fibroblasts were cultivated at 37°C in an incubator with 5% CO2, 3% O2, 95% humidity atmosphere. Cells were grown in tissue culture treated petri-dishes. Cell culture media was renewed every 2-3 days or as required. Almost confluent (80-90%) grown cells were split to new dishes to maintain the cells in log phase. For splitting of adherent cells, medium was removed and cells were washed twice with PBS. Trypsin/EDTA solution was added and the plate was incubated at 37°C for several minutes to detach the cells. Adding fresh medium inhibited trypsinization due to the presence of serum. Cells were mixed well and seeded in new dishes. DMEM medium was used for the culturing of all the adherent cells.

2.3.3 Suspension cell culture

Mouse Eμ-myc B cell lymphomas were also cultivated at 37°C in an incubator with 5% CO2, 20% O2, 95% humidity atmosphere. Cells were grown in petri dishes lined with senescent NIH 3T3 cells. To senesce NIH 3T3 cells, they were irradiated with 30 Gy of ionizing radiation and plated in tissue culture treated petri dishes as per the

protocol for adherent cell culture mentioned above. These cells were grown overnight and seeded in new culture dishes with the confluency of 10^6 cells per well of a 6 well plate in B cell medium. After another overnight incubation, allowing the cells to adhere to the plate, and release cytokines and chemokines conducive for culturing of primary murine lymphoma, lymphoma cells were added to these wells.

2.4 Cell and Tissues

2.4.1 Primary MEF isolation

Wild-type primary MEFs were isolated from E13.5 embryos by following standard lab protocol.

2.4.2 Primary lymphoma isolation

C57BL/6 background Eμ-myc transgenic mice with desired phenotype were monitored for lymphoma onset. The mice were sacrificed when the lymphomas became greater than 5mm in diameter. The enlarged lymph nodes were excised after CO2 euthanasia of the mice. Single cell suspensions were produced using the gentleMACS^{TM}, Miltenyi instrument. For histopathological analysis, the lymph nodes were snap-frozen or formalin-fixed as described previously[32,70].

2.4.3 Harvesting brain from APPPS1 transgenic mice

Heterozygous APPPS1 +/- transgenic mice (termed as APPPS1) were used as a mouse model of Alzheimer's disease. The APP and PS1 transgene refer to Thy1-APPKM670/671NL and Thy1-PS1L166P mutations, respectively. These mice were bred in the lab of Prof. Dr. Frank Heppner, Department of Neuropathology, Charité – Universitätsmedizin Berlin. The brains were harvested from these mice along with the brains of age matched APPPS1 -/- littermate controls. The brains were harvested and fixed in the lab of Prof. Heppner, and were sectioned in our lab for staining.

In these transgenic mice there is an onset of cerebral β-amyloidosis in the frontal cortex, as early as two months of age. This is followed by appearance of small amyloid plaques.

2.5 Cell transfection and infection

Low passage Phoenix cells were grown in a 10 cm petri dish up to a density of about 70%. 20 μg retroviral plasmid, 15 μg helper plasmid and 62.5 μl CaCl2 were mixed in a Polystyrene, round bottom tube and adjusted with dH_2O to up to 500 μl. To this, 500 μl 2x HBS was added dropwise, with constant agitation (gentle air bubbles). DNA precipitation occurs within 5 minutes at RT (mixture gets milky and cloudy). This precipitate was added carefully and dropwise to Phoenix cells, which have fresh 10 ml DMEM medium with 25 μM chloroquine. After 12 hour incubation, medium

including the precipitate was replaced with 4-5 ml of DMEM medium (B cell medium for lymphomas) for collecting virus supernatant.

To be transduced cells are seeded appropriately, such that they remain in the log phase throughout the transduction. The virus supernatant was collected after approximately 24 hours after the medium was replaced. 4 µg/ml polybrene was added to the supernatant and transferred to the cells to be transduced. New medium (4-5 ml) was added to the Phoenix cells for the next round of infection. The cells were incubated and grown under standard conditions.

After 12 hours of incubation, the second virus supernatant was harvested according to the procedure above, and supplemented with polybrene and added to the cells. After spinoculation of the plates (1500 rpm, 10 minutes, 32°C), cells were incubated and grown until the next round of transduction. In addition, new medium was added to the Phoenix cells for the next round of transduction. The third and fourth virus supernatants were collected 12 hours and 24 hours later according to the procedure above.

12 hours after the last transduction, old medium was removed from the cells and fresh DMEM (BCM) medium was added. Cells were grown for approximately 24 hours to allow cells to express the gene of interest. Cell population was selected with puromycin (~2 days) or blasticidin (~5 days) until non-transduced cells were completely dead.

2.6 Cell viability, Apoptosis and senescence

2.6.1 Cell number and cell viability

Cells were harvested and resuspended in a small volume of 1 x PBS (e.g. 500 µl). The trypan blue solution was added as 1:1. 10 µl was transferred to the hemocytometer and checked for equal distribution of the cells. For viability assessment and cytotoxicity assays, at least 200 cells were counted in total (dead and alive). Percentage of viability was indicated as the ratio of living cells to the whole cell number. For total cell number and growth analysis, all viable cells in 16 quadrants of the big 4-square-field were counted and number of living cells in culture was assessed by the following calculation: cell number / ml = total number of cells alive in 16 quadrants x dilution factor x 10^4.

Alternatively, cells were counted using the LUNA™ Automated Cell Counter. The cells were diluted with trypan blue, 1:1, like using the hematocytometer. 10µl of this solution was transferred to the disposable slides specific for the machine and counted according the machine's protocol.

Another method used for counting the cells was using the Guava® easyCyte flow cytometer. The manufacturer's instructions were followed for counting the cell number. The cells were diluted in the Guava ViaCount solution provided with the machine. The dilution was mentioned in the program while measuring the cells for the appropriate results.

2.6.2 Therapy-induced senescence

Lymphoma cells were seeded at a confluent density of approximately 5 x 10^5 cells per ml in a 6 well plate. Adriamycin was added to the medium to the final concentration of 0.05 µg/ml. Medium and feeder were changed after 3 days and cells were cultivated for 2 more days and harvested for further experiments.

2.6.3 Chemical inhibitors treatment

Lymphomas were treated with chemical inhibitors (14nM Semagacestat) and assayed after 24-48 hours.

2.6.4 Induction of an inflammatory response

Lymphomas were treated with a Lipopolysaccharide (LPS) concentration of 50ng/ml in serum-free medium for duration of 2 hours to induce an inflammatory response.

2.7 Growth parameters and detection of cellular senescence

2.7.1 Growth curve

Adherent cells were trypsinized from original cell culture plates. 2×10^4 MEFs were plated onto 6-well plates. In case of suspension cells, such as lymphomas, 1-2 x 10^6 cells were plated on fresh NIH 3T3 cell coated 6 well plates. Cell numbers were counted over a defined period and viable cell number was estimated by trypan blue exclusion or the Guava® easyCyte flow cytometer.

2.7.2 Senescence Associated-β-galactosidase assay

Suspension cells were collected on glass slides by cytospin. These cells temporarily adhere to the glass slides and can be fixed by carefully adding the fixation solution on top. Adherent cells were seeded in 6 or 12-well plates containing 5 mm round glass cover- slips and incubated under standard condition overnight to let the cells attach. Afterwards, medium was removed and cells were fixed in freshly prepared fixation solution.

After 15 minutes incubation, fixation solution was removed and cells were washed twice in PBS. Staining solution was added and plates were transferred and incubated in a humidified atmosphere at 37°C for 16-20 hours. Cells were gently washed with PBS and mounted afterwards using Mowiol® 4-88.

2.7.3 Cell cycle BrdU/ PI FACS analyses

20 µM BrdU was added in the cell culture medium 2 hours before harvesting cells. Afterwards, cells were trypsinized, pelleted, washed with PBS. Cells were fixed by dropping 5 ml 80% ice-cold ethanol, while simultaneous vortexing and incubated in 4°C for at least 24 hours. Furthermore, the cells were washed and resuspended in 2M HCl and incubated at room temperature for 30 minutes. Post centrifugation, cells were treated with 0.1M Na-borate for 15 minutes and washed with PBST. Next, the cells were incubated with anti-BrdU- FITC antibody in the dark, overnight. Following day, cells were incubated with 100 µg/mL propidium iodide (in PBS) and analyzed by FACS.

2.8 Immunoassays

2.8.1 Immunoblotting (western blot)

30~60 µg of protein samples were loaded in polyacrylamide gel and gel was run at 80 volts (120 volts after proteins pass through the stacking gel) in 1x SDS running buffer. After electrophoresis, proteins were transferred to PVDF membrane using a semi-dry transfer chamber (BioRad). After the transfer was complete, the membrane was blocked with 5% milk in PBST for 1 h and then incubated with appropriately diluted primary antibody solution (5% BSA in PBST) at 4°C overnight. The blot was washed 3 times 10 minutes with PBST and incubated with appropriate secondary antibody for 1 h at room temperature. The blot was again washed with PBST and chemiluminescent peroxidase substrate was applied on the membrane. Images were visualized and taken by Intas Chemocam Imager or ChemiDoc MP Imaging System.

2.8.2 Immunofluorescence (IF)

Adherent cells were seeded in 6 or 12-well plates containing 5 mm round glass cover-slips and incubated under standard condition overnight to let the cells attach. Suspension cells were attached to the glass slides by cytospin. Cells were fixed with 4% PFA for 15 minutes and washed 2 times with PBS. Then cells were permeabilized in 0.2% Triton X-100/PBS for 15 minutes and blocking with 1% BSA/PBS. After the incubation with primary antibody overnight (4°C), cells were washed 3 times 10 minutes with PBST, and subsequently incubated with fluorescence-labeled secondary antibody for 60 minutes in the absence of light. Cells were washed with PBST, mounted with ProLong™ Diamond Antifade Mountant or and fluorescence was detected using Leica SPE fluorescent microscope or Keyence BZ-9000 fluorescence microscope. Slides were stored in the dark.

2.8.3 Immunohistochemistry (IHC)

Cells or tissue sections were fixed, permeabilized, blocked and incubated with primary antibody as described above (IF staining). On the next day, after washing with PBST, cells were stained with Dako REAL™ Detection System according to the manufacturer's instruction.

2.8.4 Amyloid staining

1.4mM stock solution of pFTAA, prepared in dH_2O, was stored in -20°C. Working dilutions of 1:500 in PBS were used. The brain sections were prepared on glass slides using optimal cutting temperature compound (OCT compound). The sections were washed three times in 1x PBS to remove the cryoprotectant and incubated for 30min in pFTAA staining solution at the working dilutions. These sections were again rinsed with 1x PBS and covered using the aqueous mounting agent Aquatex. These sections were analyzed the following day.

2.8.5 Co-staining with pFTAA

Staining with pFTAA was done after any other staining or assay that was combined with this staining (for e.g. Senescence Associated-β-galactosidase assay or immunostainings), as per their respective protocols.

2.8.6 Quantification of Aβ by an electroluminescence linked immunosorbent assay system

Intracellular and extracellular $A\beta_{40}$ and $A\beta_{42}$ concentrations in the control lymphoma cells were determined with an electroluminescence linked immunosorbent assay system (MSD assay) using the MSD 96- Well MULTI-SPOT® Human (4G8) Abeta Peptide Panel 1 V-plex Assay (MSD, Meso Scale Discovery). The Assay was performed using the instructor's manual.

2.9 Quantitative real-time PCR

For quantitative RT-PCR analyses (RQ-PCR), RNA was extracted with TRIzol (Invitrogen) and transcribed into cDNA using SuperScriptIII reverse transcriptase (Invitrogen) primed by oligo-dT according to manufacturer's protocol. RQ-PCR analyses of candidate transcripts were conducted using commercially available primers (Applied Biosystems). Calculation of individual transcript expression levels relative to the expression of GAPDH as a housekeeping control mRNA has been described previously[160].

RESULTS

1 Induction of senescence in Eμ-*myc* lymphomas using chemotherapy

To study TIS, we used Eμ-*myc*-driven B-cell lymphomas as a model system, that overexpress the anti-apoptotic protein B-cell lymphoma 2 (Bcl2) via retroviral infection, referred to as control;bcl2 lymphomas from here on. Induction of DNA damage response using the chemotherapeutic drug Adriamycin (ADR, a.k.a. doxorubicin), resulted in TIS by evading apoptosis[161,162] (Figure 5). Senescent cells exhibit different features such as senescence-associated-β-galactosidase (SA-β-gal) activity[10], absence of the S phase detected by Bromodeoxyuridine (5-bromo-2'-deoxyuridine)/Propidium Iodide (BrdU/PI) staining and up regulated protein expression of various markers of senescence detected by immunoblotting (Figure 6).

SA-β-gal is a hydrolase enzyme mediates the hydrolysis of β-galactosidases to monosaccharides, which is a characteristic of senescent cells[10]. For visualization, a chromogenic substrate X-gal is utilized which results in the production of a blue dye precipitate[163] (Figure 6A). BrdU, on the other hand, is used for detecting proliferation. It is incorporated into the DNA during the S phase of the cell cycle, since BrdU is an analog of thymidine. With an anti-BrdU antibody, cells that are actively replicating their DNA, thereby incorporated BrdU, can be detected. These cells are usually analyzed by flow cytometry, along with DNA dyes such as PI stain to observe cells in the various phases of cell cycle (Figure 6B). Senescent cells are mostly arrested in the G1 phase and S-phase cells disappear. As mentioned above, there are several proteins that have a defined role in senescence and therefore can be used as markers of cellular senescence. These include phosphorylation of p53- serine 15, p21 and trimethylation of H3K9 (Figure 6C).

Observing a combination of all these senescence features is used in this thesis to confirm cellular senescence.

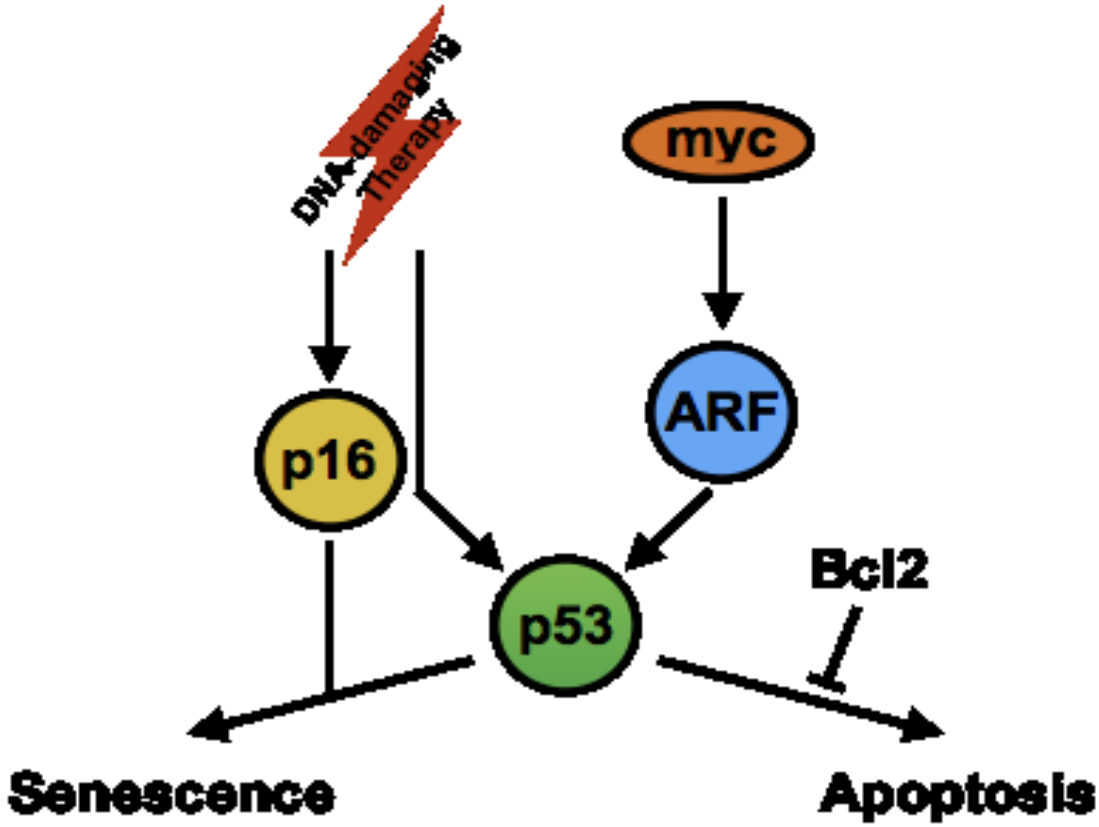


Figure 5: Therapy-induced senescence in Eμ-*myc*-driven B-cell lymphomas. Senescence induction using a chemotherapeutic agent such as ADR or CTX induce DNA damage, resulting in TIS. TIS is controlled by p53 and/or $p16^{INK4a}$. These anticancer agents cause activation of p53 independent of ARF, as loss of function mutation or loss of p53 or INK4a leads to senescence defect, while deficiency of ARF alone is unable to disrupt senescence. Therefore, induction of senescence by a DNA damaging procedure leads to an upregulation of p53 and $p16^{INK4a}$, probably via a common mechanism. Anti-apoptotic gene *Bcl2* prevents the cells from undergoing apoptosis in response to DNA damage and bypasses the cells into senescence. Figure adapted from Schmitt et al. 2002[53].

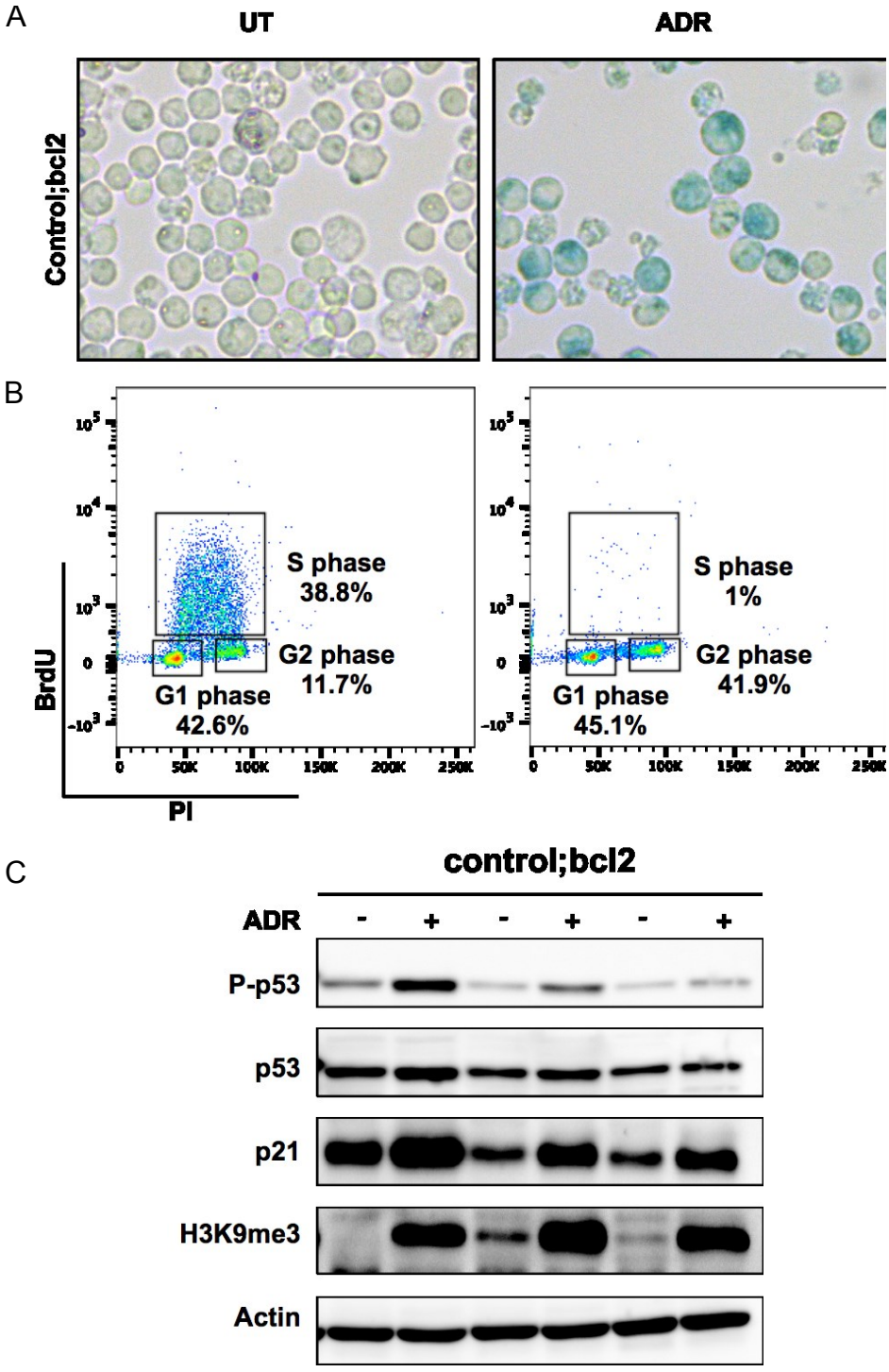


Figure 6: Markers of senescence. (A) The increased lysosomal activity that is characteristic of a senescent cell, observed by SA-β-gal assay. (B) BrdU/PI staining, where the S phase of the cell cycle disappeared, observed 5 days after treatment. (C) The expression levels of senescence markers were observed by immunoblotting.

2 Senescence presents with features reminiscent of Alzheimer Disease

2.1 Therapy-induced senescence is associated with AD genes

2.1.1 Affymetrix data revealed an upregulation of AD genes in TIS capable cells

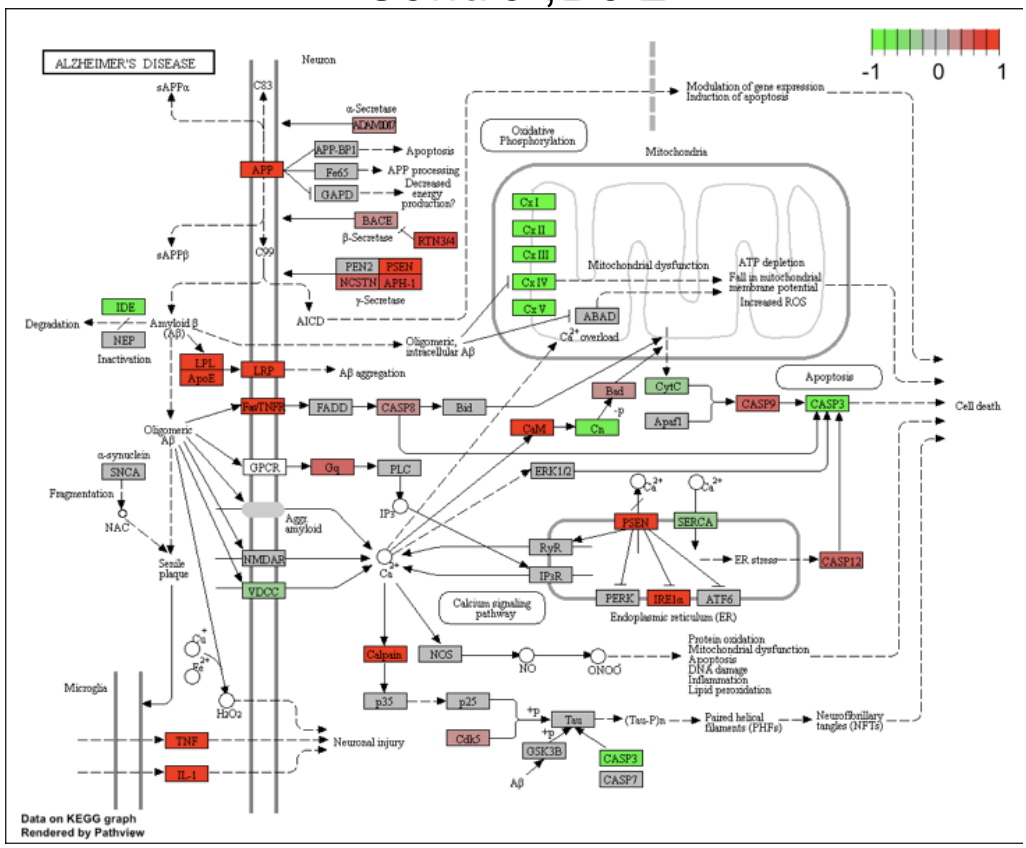
Affymetrix Mouse Genome 430 2.0 Array using 15 matched pair control;bcl2 lymphomas was performed by colleagues from the lab. The enriched pathways in TIS lymphomas were determined. Upon screening the KEGG pathway database, genes of the AD pathway were enriched in TIS lymphomas. AD pathway according to the KEGG database presents with genes that contribute to the pathology of AD. This database was used to define a set of genes- termed collectively as the “AD machinery or AD genes”.

By utilizing the Bioconductor pathview on R, the individual AD genes that have an altered expression after TIS compared to their untreated control;bcl2 lymphoma matched pair, were depicted (Figure 7A). When senescence was induced in control;bcl2 lymphomas after ADR treatment, many AD genes were up regulated (represented in red) and some were down regulated (represented in green). Some of the genes of the AD pathway appeared to be unchanged post TIS.

On the other hand, lymphomas which are incapable of undergoing senescence, such as the Suv39h1$^{-/-}$;bcl2 lymphomas, did not show the same degree of change in the expression of AD genes after treatment (Figure 7B). However, the threshold of detection is a limitation of this analysis. There is a saturation point to detect the level of alteration in expression. Beyond a certain level of up regulation or down regulation, change in color intensity cannot be observed. Therefore, it is possible that if there is a certain gene that appears altered in both control and Suv39h1$^{-/-}$;bcl2 lymphomas, the degree of alteration could vary compared to the other. Further quantitative analyses were carried out to understand these differences in detail. Additionally, it is possible that some players of AD could be acting upstream or downstream of the “senescence pathway”. Hence, in the case of senescence capable or incapable lymphomas, some of the AD genes could still be involved in the DDR and would be equally altered in both the cases.

A

Control;Bcl2



B

Suv39h1-/-;Bcl2

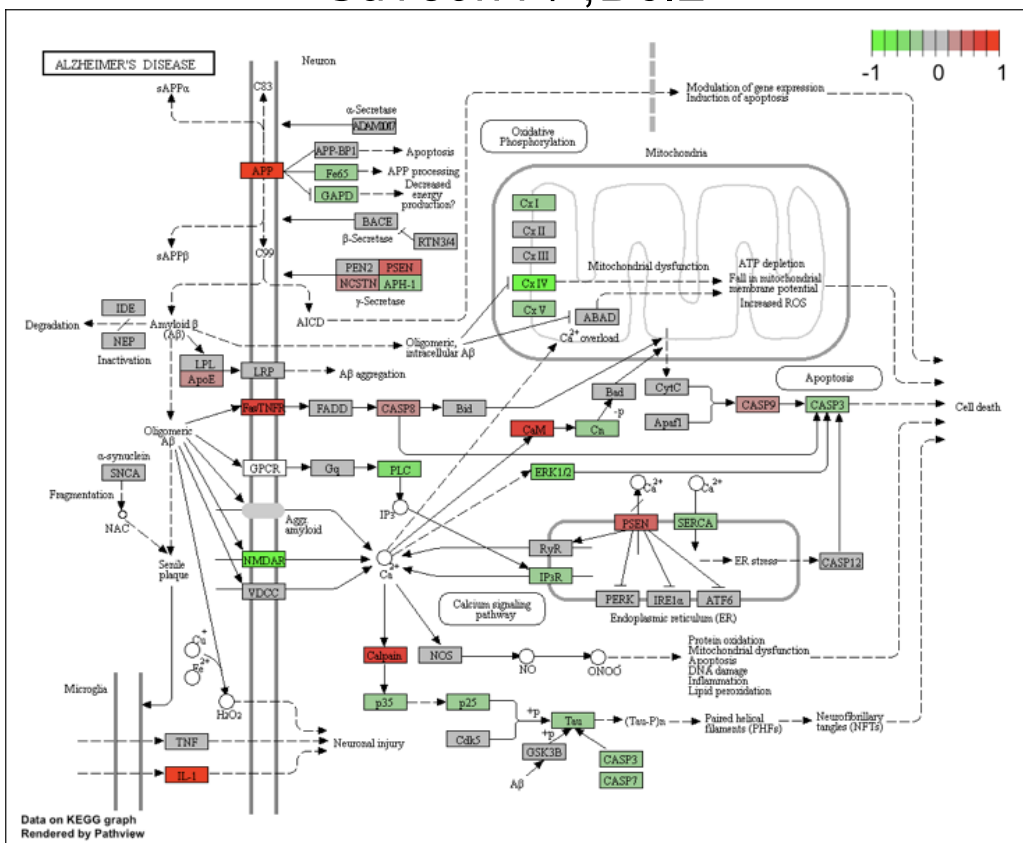


Figure 7: KEGG pathway map of Alzheimer's disease representing the up or down regulation of AD genes UT *vs* ADR treated lymphomas. (A) Control;bcl2 lymphomas TIS/UT gene expression of the AD machinery. (B) Senescence incapable Suv39h1$^{-/-}$;bcl2 lymphomas ADR/UT gene expression of the AD machinery. The up regulation is depicted by red tiles and down regulation is depicted by green tiles.

To determine if there are any overlapping features between TIS and AD, the AD gene set defined from the gene expression data set from the 15 match paired control;bcl2 lymphomas and 5 match paired Suv39h1-/-;bcl2 lymphomas was used for gene set enrichment analysis (GSEA) (Figure 8). An enrichment of the AD genes in ADR treated lymphomas, normalized to the corresponding untreated cells, was observed in the GSEA plot. The AD genes were enriched in TIS control;bcl2 lymphomas (Figure 8A, left) but not in the ADR treated senescence incapable Suv39h1$^{-/-}$;bcl2 lymphomas (Figure 8A, right). The heat map represents the expression pattern of these individual genes in the individual matched pair control;bcl2 lymphomas (Figure 8B, left). The AD related genes in the control;bcl2 lymphomas cluster into two distinct groups, up regulated or down regulated AD genes post-treatment. However, in case of the senescence incapable Suv39h1-/-;bcl2 lymphomas, ADR treatment did not result in a clear pattern of up or down regulation of these genes (Figure 8B, right).

Since the upregulated AD genes were enriched in TIS capable lymphomas but not in senescence incapable lymphomas, this suggests a biological overlap between TIS and AD. Moreover, it is intriguing that TIS in lymphomas has an impact on the expression of AD genes, since there is no known function of some of these genes, e.g. APP, in the context of lymphomas or TIS.

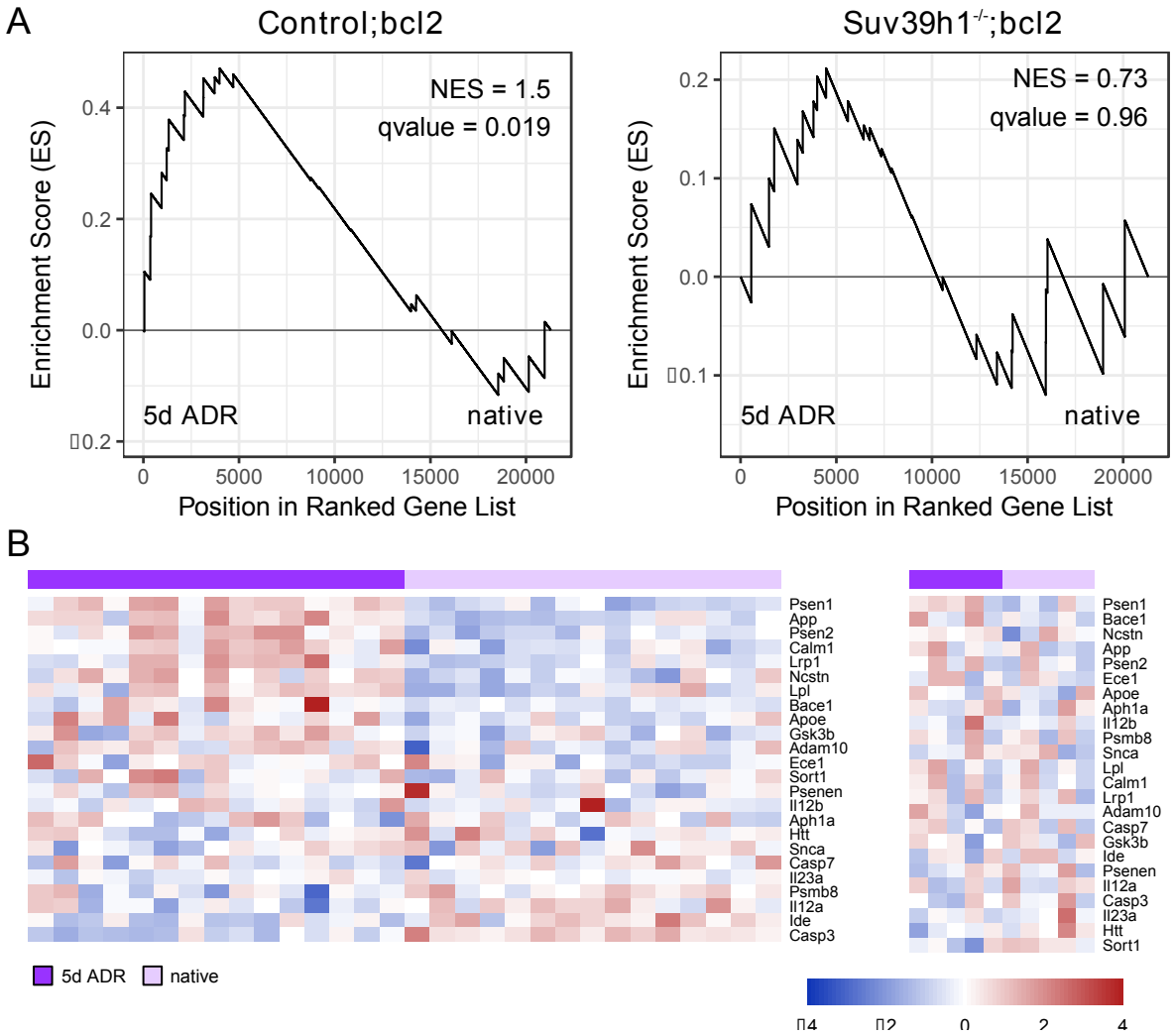


Figure 8: AD gene distribution in Eμ-myc-driven B-cell lymphomas UT (native) *vs* TIS. Genes involved in Alzheimer's disease were significantly altered in TIS control lymphomas. (A) The GSEA of Alzheimer's disease targets shows their enrichment (represented by Normalized enrichment score, NES) in senescence capable control;bcl2 lymphomas (n=15) but not in senescence incapable Suv39h1$^{-/-}$;bcl2 lymphomas (n=5) in the gene expression plot of UT *vs* ADR treated setting. (B) Heat maps show individual gene expression of individual lymphomas, UT *vs* ADR treated.

Table 1: AD genes. Alzheimers related genes derived from the KEGG pathway, which are up regulated in TIS control;bcl2 lymphomas.

Up regulated AD genes in TIS
Psen1
APP
Psen2
LRP1
Calm1
Ncstn
LPL
Apoe
Gsk3b
Adam10
Ece1
Sort1
Psenen
Aph1a
Casp7
IL6

2.1.2 Taqman® based real time PCR validation of Affymetrix data

All the above-mentioned data analysis is representative of the high throughput data from the microarray affymetrix dataset. Therefore, as a validation, the expression of the up regulated genes involved in KEGG Alzheimer's disease gene set were analyzed using Taqman® based real time PCR (RQ-PCR). Changes in the expression of these genes were observed 5 days post ADR-treatment. The AD genes with significant up regulation in the heat map (Figure 7A) exhibited similar trends via RQ-PCR (Figure 9B). Interestingly, when a set of Suv39h1$^{-/-}$;bcl2 lymphomas were analyzed for change in expression of the same genes after treatment, their expression levels, in most cases, were much lower as compared to the control;bcl2 lymphomas (Figure 9B). This confirmed that there is indeed more relevance of the AD in the context of senescence.

2.2 Proteotoxicity and SASP may be the link between TIS and AD

Proteotoxic stress has been reported in both AD and senescence[70,159]. Therefore, to determine whether AD machinery is only involved when protetoxic stress correlates with senescence, senescence was induced by rapamycin (an mTOR inhibitor)[164]. Rapamycin-induced senescence is accompanied by autophagy induction but lacks a SASP response. As a result, it is not associated with proteotoxicity-driven ER stress[70]. Treatment with rapamycin induced senescence to an extent which was comparable to TIS upon ADR treatment[70] (Figure 9A). Interestingly, in this setting the AD genes were unaffected, suggesting their association with proteotoxic stress (Figure 9B). This result further led us to pursue this project, to understand the link between senescence and AD, a disease with defective proteostasis.

Additionally, as discussed above, SASP is a NF-κB-driven phenomenon[58]. SASP leads to an increased protein production in senescence, which requires for an elevated autophagy to degrade the misfolded SASP components. Therefore, to understand if SASP related proteotoxicity is the link between senescence and the AD machinery, control;bcl2 lymphomas were stably transduced with NF-κB super-repressor, IκBαΔN[165]. These lymphomas maintain their ability to undergo TIS, *in vitro*, in response to ADR treatment[160]. Post-TIS, they have been shown to express proteins only slightly above the baseline of the non-senescent control;bcl2

lymphomas[70]. Likewise, senescent NF-κB-repressed cells express SASP cytokines at a lower level[160]. On analyzing expression of AD genes in the ADR-induced TIS of NF-κB-repressed cells, the AD genes were also expressed at a lower level, when compared to the ADR induced TIS of control;bcl2 lymphomas (Figure 9B). Confirmingly, this suggests that reduced SASP cytokine production also leads to a lower expression of the AD genes.

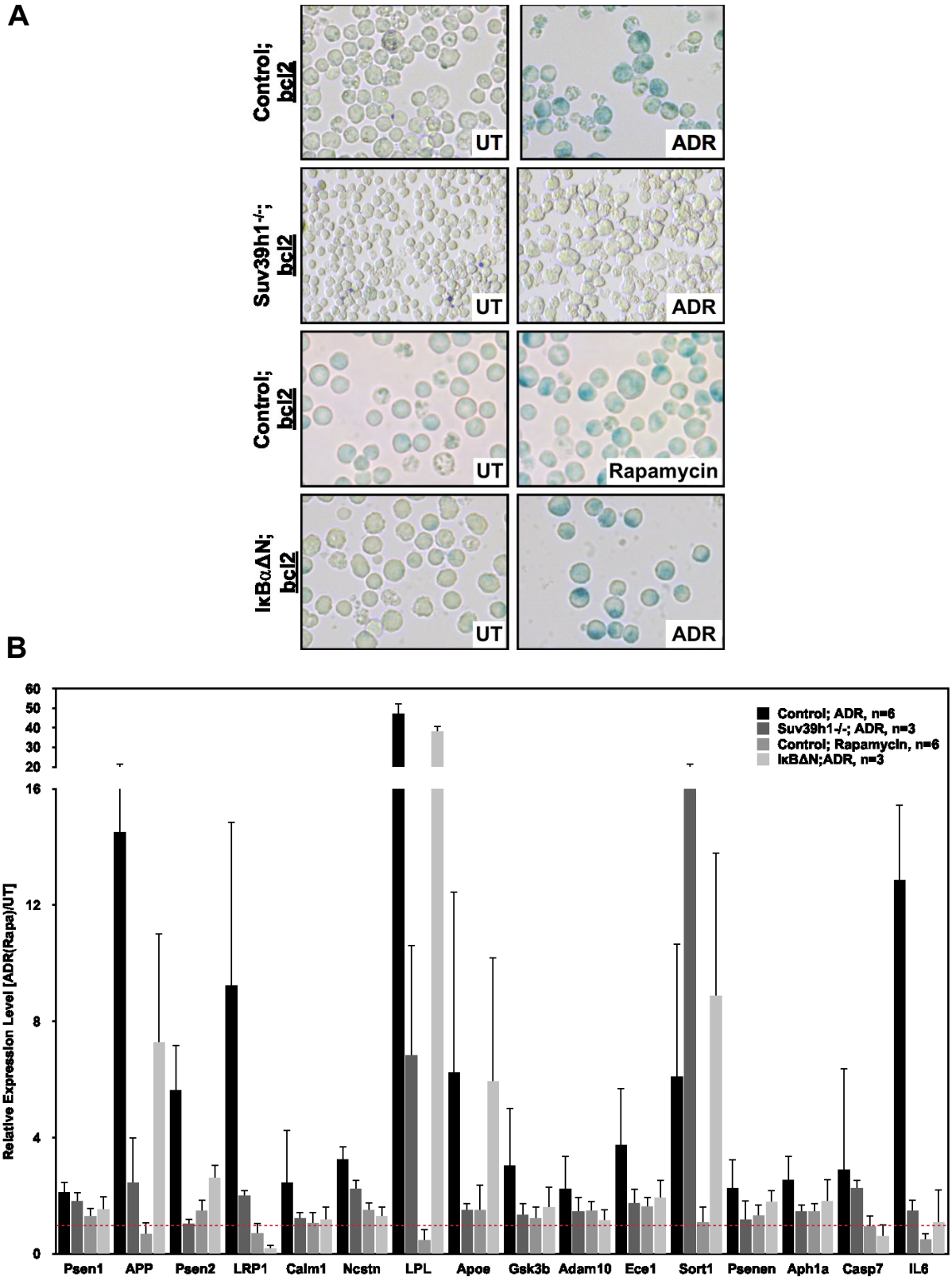

A
Control; bcl2
Suv39h1-/-; bcl2
Control; bcl2
IκBαΔN; bcl2
UT
ADR
UT
ADR
UT
Rapamycin
UT
ADR
B
Relative Expression Level [ADR(Rapa)/UT]
Control; ADR, n=6
Suv39h1-/-; ADR, n=3
Control; Rapamycin, n=6
IκBΔN;ADR, n=3
Psen1
APP
Psen2
LRP1
Calm1
Ncstn
LPL
Apoe
Gsk3b
Adam10
Ece1
Sort1
Psenen
Aph1a
Casp7
IL6

Figure 9: AD gene expression in various TIS settings (A) SA-β-gal confirms senescence in different settings and treatments. (B) RQ-PCR analysis of the indicated AD genes that are up regulated, as in Figure 8. The relative expression levels are normalized to the control cells carrying the vector, that are comparable throughout all datasets. Fold change of the relative expression levels, compared to the UT matched pair lymphoma, are indicated in the histogram. The relative expression levels of the UT lymphoma was set to 1 (indicated by the red line). IL6 represents a SASP factor, which is a secreted NF-κB target. All histogram bars indicate mean values ± standard deviation.

Therefore, from these experiments we infer that there is an involvement of AD genes in the context of senescence. These genes were up regulated when TIS was induced in control;bcl2 lymphomas by ADR but not by Rapamycin treatment. This suggests that the role of AD genes in the context of TIS could be the underlying proteotoxic stress. Moreover, TIS in the NF-κB-repressed cells, thereby the SASP-repressed cells, also resulted in lower expression of the AD genes as compared to the controls. However, not all the AD genes were completely repressed in TIS of the NF-κB-repressed cells, e.g. LPL, LRP1, APP, and SORT1, but their expression was, for the most part, many folds lower in comparison. This could be due to a repressed but not completely absent SASP in these cells, resulting in some proteotoxic stress. Besides, these genes could also have other unidentified functions in senescence.

Hence, it can be concluded that SASP, being a major contributor of proteotoxicity in a senescent cell, could possibly be the common denominator between AD and TIS.

2.3 APP and Psen1 are not induced as an inflammatory response

It was observed that AD machinery was only up regulated in the case of TIS after ADR treatment, which includes SASP (Figure 9). SASP involves secretion of inflammatory factors that might facilitate induction of AD machinery in TIS lymphomas, either through an inflammation specific response or a senescence specific response.

To determine this, lymphomas were treated with lipopolysaccharide (LPS) to induce an inflammatory response. LPS is a large molecule, consisting of a lipid and a polysaccharide. It acts as an endotoxin since it can bind to the CD14/TLR4/MD2 receptor complex and stimulate cells such as B cells, macrophages, etc., to secrete pro-inflammatory cytokines[166–168]. Induction of an

inflammatory response was confirmed by analyzing the change in the expression levels of IL6 and IL1a by RQ-PCR (Figure 10). Even though there was a significant change in the mRNA expression levels of IL6 and IL1a, mRNA expression levels of the representative players of AD- APP and Psen1, were not affected. This suggests that the link between AD machinery and senescence is SASP and not inflammation, per se.

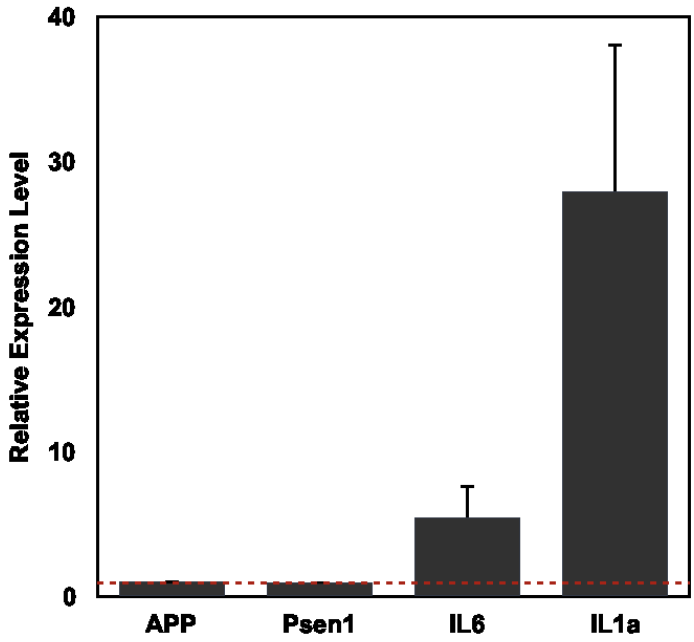


Figure 10: Inflammatory response and AD. Control;bcl2 lymphomas were treated with LPS in order to induce an inflammatory response. This was confirmed by the increase in the expression levels of IL6 and IL1a, which are also secreted targets of NF-κB. The expression levels of a few representative targets of the AD machinery, APP and PSEN1 were also observed. The relative expression levels of the UT lymphoma was set to 1 (indicated by the red line). All histogram bars indicate mean values ± standard deviation. (n=4)

2.4 Oncogene-induced senescence does not involve AD gene upregulation

Activation of the alzheimers genes after TIS in control;bcl2 lymphomas suggested that there is probably some unearthed role of this machinery in senescent cells. But whether the involvement of the AD machinery was restricted to TIS or if could also be a component of other forms of senenscence was not yet clear. Thus, to address this, OIS which is another major senescence type involved in tumor progression, was explored.

Senescence was induced in MEFs by over expressing RasG12V mutant in the wild type cells. These cells undergo OIS due to the DNA damage. After RasG12V was successfully induced in MEFs, the cells typically show senescence markers within 10 days (Figure 11A), following which the expression of the AD genes were analyzed. Interestingly, this type of senescence did not result in the up regulation of AD genes (Figure 11B), suggesting that this machinery is not involved in all types of senescence.

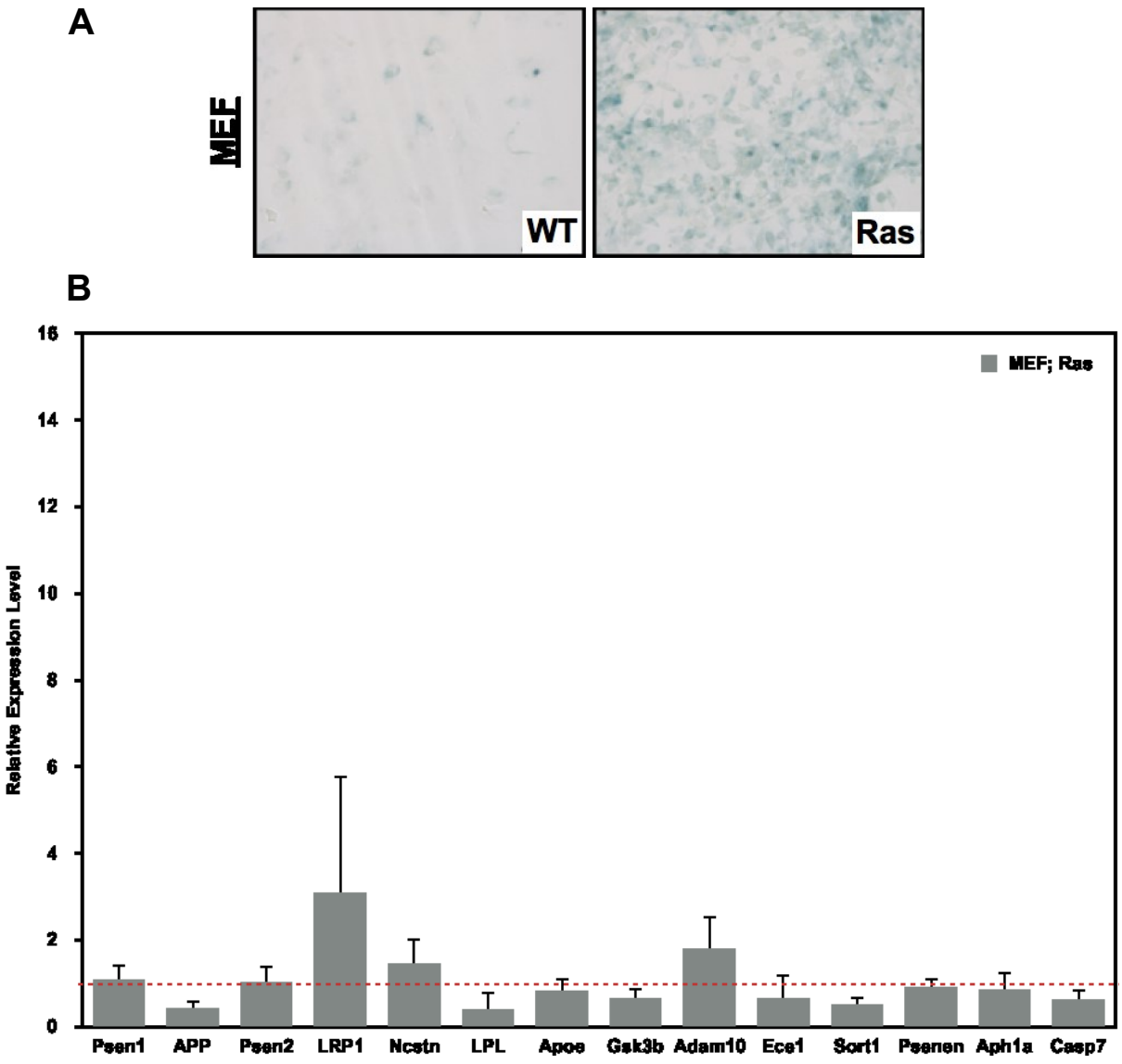


Figure 11: AD gene expression in OIS. (A) OIS was induced in MEFs by overexpression of the RasG12V oncogene. Senescence was confirmed 10 days after infection, by SA-β-gal. (B) The expression analysis of the same subset of the AD genes as in TIS was analyzed. The relative expression levels of the WT MEFs was set to 1 (indicated by the red line). All histogram bars indicate mean values ± standard deviation. (n=4)

3 AD model systems exhibit features of senescence

3.1 Brain of *APPPS1*$^{+/-}$ transgenic mice indicate co-occurrence of senescence and Aβ plaques

Recent reports strongly suggest a role for senescence in the etiology of AD[169]. The underlying mechanism however is not well understood. Here, we used different AD model systems and investigated various features of senescence associated with them.

To understand the association between senescence and AD, from an AD viewpoint, the brains of an AD mouse model were analyzed. These mice are double transgenic for APP and Psen1 (*APPPS1*$^{+/-}$). They start to develop AD characteristics, such as Aβ plaques, from as early as 2 months of age. Cognitively, they exhibit impairment in behavorial assessments such as memory tests, by the age of 8 months[170]. The presence of Aβ plaques in their brains was visualized by the pFTAA dye (Figure 12A), because pFTAA dye has an affinity to bind to the conformational structure of these plaques[171].

As expected, the older (330 days) transgenic mice developed a prominent plaque phenotype when compared to the younger (69 days) transgenic mice (Figure 12A). This phenotype is gender independent as equal numbers of female and male mice were analyzed with similar results. Moreover, the age-matched wild type C57BL/6 control mice did not show any Aβ plaques in the brain sections of neither younger nor older age groups. These results confirmed the involvement of APP and Psen1 in plaque generation.

Interestingly, SA-β-gal staining of the same brain sections demonstrated that the older transgenic mice present with a higher number of prominent blue cell clusters, as compared to younger mice. The age matched wild type mice brains did not show any significant staining (Figure 12B). This indicates that in *APPPS1*$^{+/-}$ transgenic mice, increasing age not only results in higher number of Aβ plaques but also causes higher level of senescence in their brains. The young transgenic mice reiterated this observation with an exhibition of much lower number of plaques correlating with lower level of senescence. Moreover, the Aβ plaques and senescent

cells exhibited co-localization, with senescent cells largely present in the peripheral regions of the plaques (Figure 12C). This suggests that APP and Psen1 are not only relevant for plaque generation, but also have a direct or indirect involvement in the senescence induction.

More number of senescent cell clusters was found to be present in the brain of younger transgenic mice as compared to the plaques, suggesting a possibility that senescence precedes plaque formation. Taken together, these results provide strong circumstantial evidence for the involvement of senescence in AD related plaque formation.

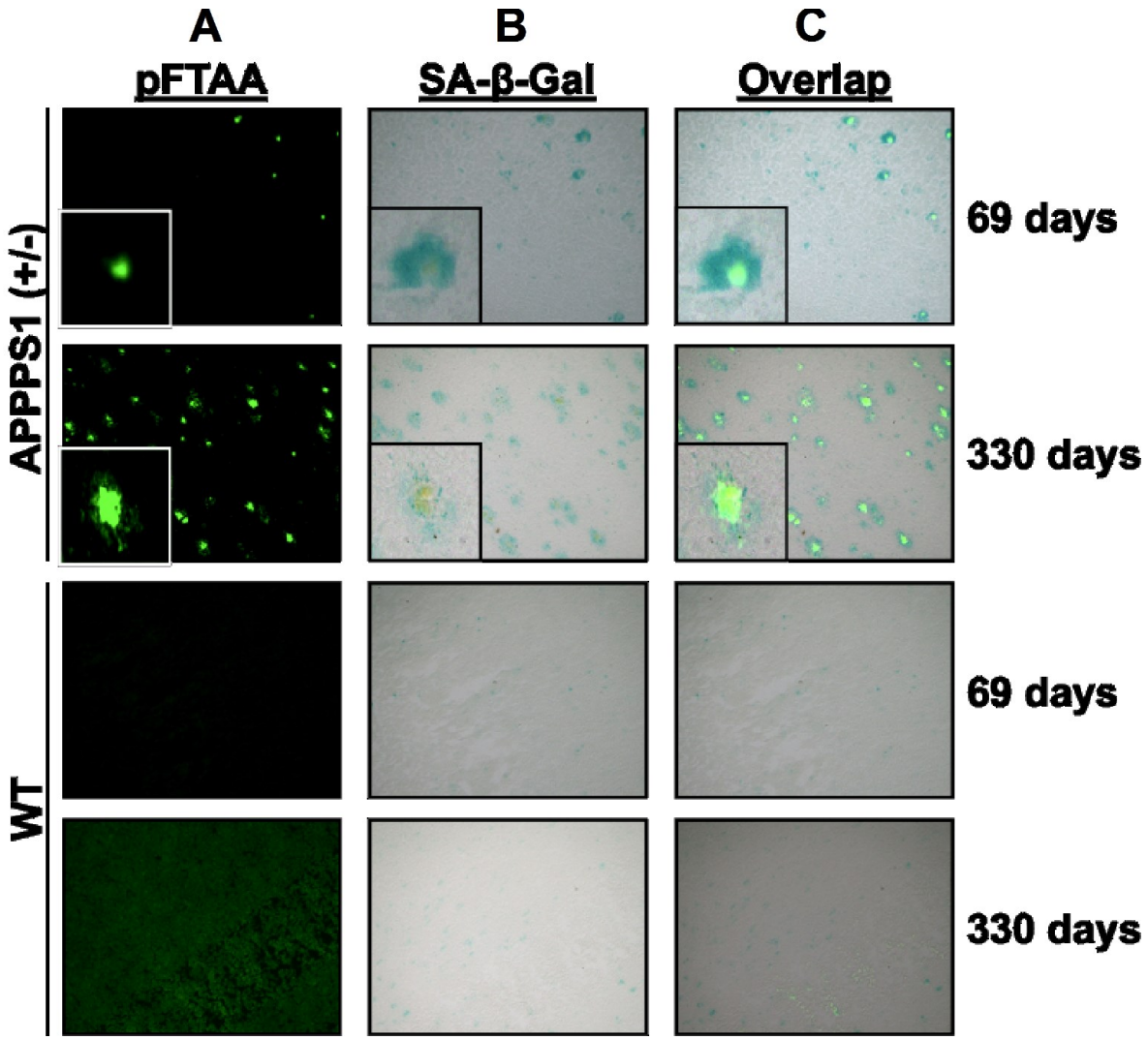


Figure 12: Correlation between senescence and Aβ plaque. Brain sections of *APPPS1*$^{+/-}$ transgenic mice, with age groups of 69 days (n=4) and 330 days (n=3) were double stained for extracellular Aβ plaques and senescence. Same number of age matched C57BL/6 wildtype (WT) mice were used as controls. (A) Aβ plaques in the brains of mice differing in age or genotype were analyzed. Higher numbers of plaques were observed in 330 days old transgenic mice, compared to the 69 days old mice. The WT mice showed no detectable plaques up to 330 days of age. (B) These images describe senescence in the brains of the mice. The number of senescent cell clusters observed in these brain sections was directly proportional to the Aβ plaques. (C) Co-localization of images (A) and (B) revealed that senescent cells were present around the Aβ plaques.

3.2 Aβ plaques were not detected in TIS lymph nodes

We observed that TIS in control;bcl2 lymphomas show up regulation of APP transcripts, quantified by RQ-PCR. This led us to the next question, whether TIS lymph nodes present with Aβ plaques, as in the brain of *APPPS1*$^{+/-}$ mice.

To achieve this, mice bearing lymph nodes were treated with cyclophosphamide (CTX). TIS was confirmed 7 days post treatment, by SA-β-gal assay of lymph node sections. These sections were co-stained with Aβ antibody for detecting Aβ plaques by immunohistochemistry. It was observed that in these senescent lymph nodes, Aβ plaques were not formed (Figure 13). Similarly, no plaques were observed when pFTAA dye was used for the detection of Aβ plaques (data not shown).

This implies there are no Aβ plaques formed in the lymph nodes of control;bcl2 mouse models. It is possible that lymphoma cells do not release Aβ to form plaques. Alternative explanations could be that 7 days is insufficient to form plaques in the lymph node or may be the Aβ that is released by the cell is washed away due to a flow through of lymph through the lymph node.

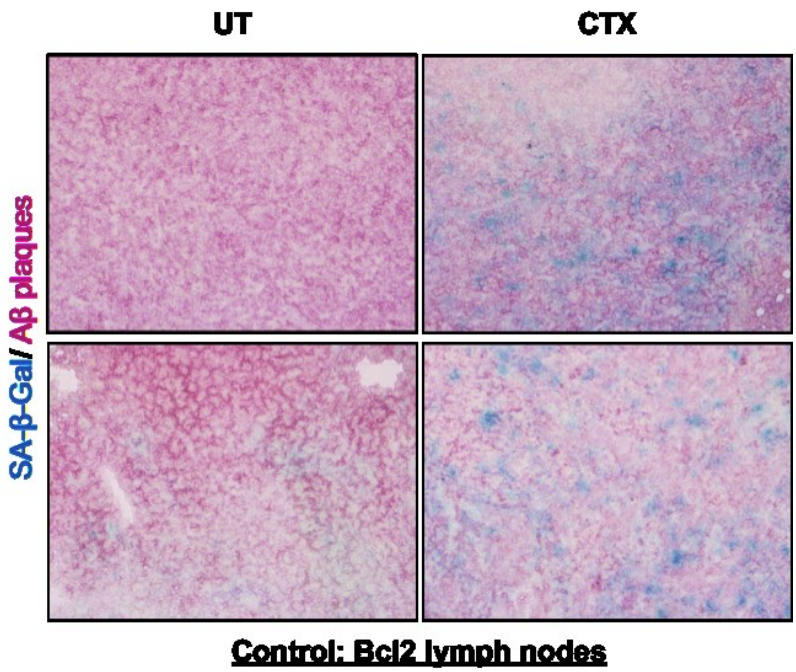


Figure 13: Aβ plaque detection in lymph node sections post TIS. Lymph node sections from pairs of mice (n=4) transplanted with the same lymphomas. One mouse was exposed to treatment with cyclophosphamide (CTX) whereas the other mouse was used as a control (UT). The sections were double stained with SA-β-gal (blue) followed by IHC for Aβ plaques (red).

3.3 APP contributes to ER stress and autophagy along with other markers of senescence

As previously mentioned, there are certain proteins, including p53 and p16, which are strong mediators of cellular senescence. These proteins have been demonstrated to be indispensable in senescence, as their absence potentially allows cells to escape senescence[32,172,173].

Swedish mutation of APP is one of the well-known genetic mutations leading to EAOD (FAD)[174]. Many mouse models carrying this mutation are used to study genetic forms of AD. Interestingly, overexpression of Swedish mutation of APP in a neuroblastoma cell line SH-SY5Y, a cell line model for AD, resulted in an increased expression of many of these senescence marker genes (Figure 14): phosphorylation of p53, induction of p21, increased tri-methylation of H3K9, etc. Furthermore, it was observed that the overexpression of APP also induced ER stress (detected by the increased p62 (or p62/SQSTM1) expression) as well as a burden on the autophagic-lysosomal machinery (detected by the increased cleavage of MAP1-LC3-I-isoform to MAP1-LC3-II-isoform). This reiterated the findings of Yang and colleagues, where they described that in an APP transgenic mouse model there is an accumulation of undigested autophagy substrates like p62 and MAP1-LC3-II-isoform, thereby hinting towards compromised autophagic-lysosomal machinery[175].

Although over expressing APP in SH-SY5Y cells led to an up regulated expression of many proteins involved in senescence induction, it was not sufficient to induce senescence in this cell line (observed by growth curve and SA-β-gal assay, data not shown). This could be because these cell lines are immortal and can overcome the stresses caused by over expression of these proteins.

Therefore, from these results we can infer that in addition to AD, APP also contributes towards selective features of senescence, such as up regulation of proteins that are either regulators of senescence or downstream to them. This argues for a potential role for APP in senescence biology.

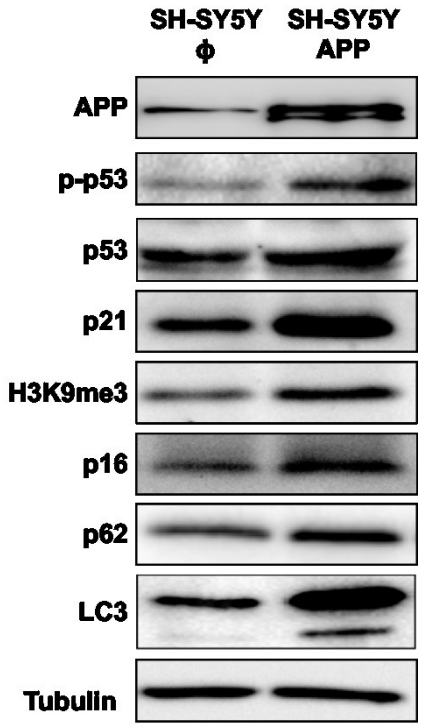


Figure 14: Consequences of APP overexpression on markers of senescence
Swedish mutation of APP was overexpressed in SH-SY5Y cell line. This led to an induction of many markers of senescence, depicted by immunoblot analysis. Tubulin was used as a loading control (n=3).

4 APP over expression does not make a cell susceptible towards TIS

Figure 14 demonstrated that the neuroblastoma cell lines over expressing APP led to an up regulation of the senescence markers, indicating a possible pre-existing senescence burden in the APP overexpressing neuroblastoma cells. Since, this burden was not sufficient to induce senescence in these cells, therefore, the next question that arises is whether it predisposes them towards TIS.

To explore this, the SH-SY5Y cells (parental and Swedish APP) were treated with ADR, similar to control;bcl2 lymphomas. A change in cellular morphology was observed, as the cells appeared more flattened. However, about 60% of the viable cell population was rendered senescent 5 days post treatment, irrespective of the APP status, confirmed by SA-β-gal staining (Figure 15A). Likewise, there was a sharp increase of G1 phase cells in treated cells (66.1%) as compared with the untreated cells (48.3%) but not all cells were arrested as indicated by S-phase fractions with positive BrdU staining (Figure 15B). This difference might be due to suboptimal levels of ADR dosage or treatment time.

When the growth of these cells was monitored (Figure 15C), we observed that the APP overexpressing SH-SY5Y cells grow at a slower rate as compared to the parental cells. This could be due to the increased proteotoxic or other burdens of these cells. Post treatment, the viable cell population was reduced to half after 3 days (Figure 15C, lower box). This revealed that initially ADR treatment induced death of around 50% of the cell population. The remaining cells were partly senescent and partly non-senescent. The non-senescent cells appear to proliferate albeit at a much slower rate as compared to the untreated cells. Both the parental and the Swedish APP variants of SH-SY5Y cells exhibited similar outcomes. Varying concentrations of ADR (10 or 100 fold) were also used to induce senescence in these cell lines but they proved to be highly toxic (data not shown).

In conclusion, on one hand, using the neuroblastoma cell line as a model system of AD exhibited a mechanistic correlation between APP and some markers of senescence. But on the other hand, they don’t seem to be an appropriate model

system of TIS, due to their inability to undergo TIS homogenously via the standard treatment applications.

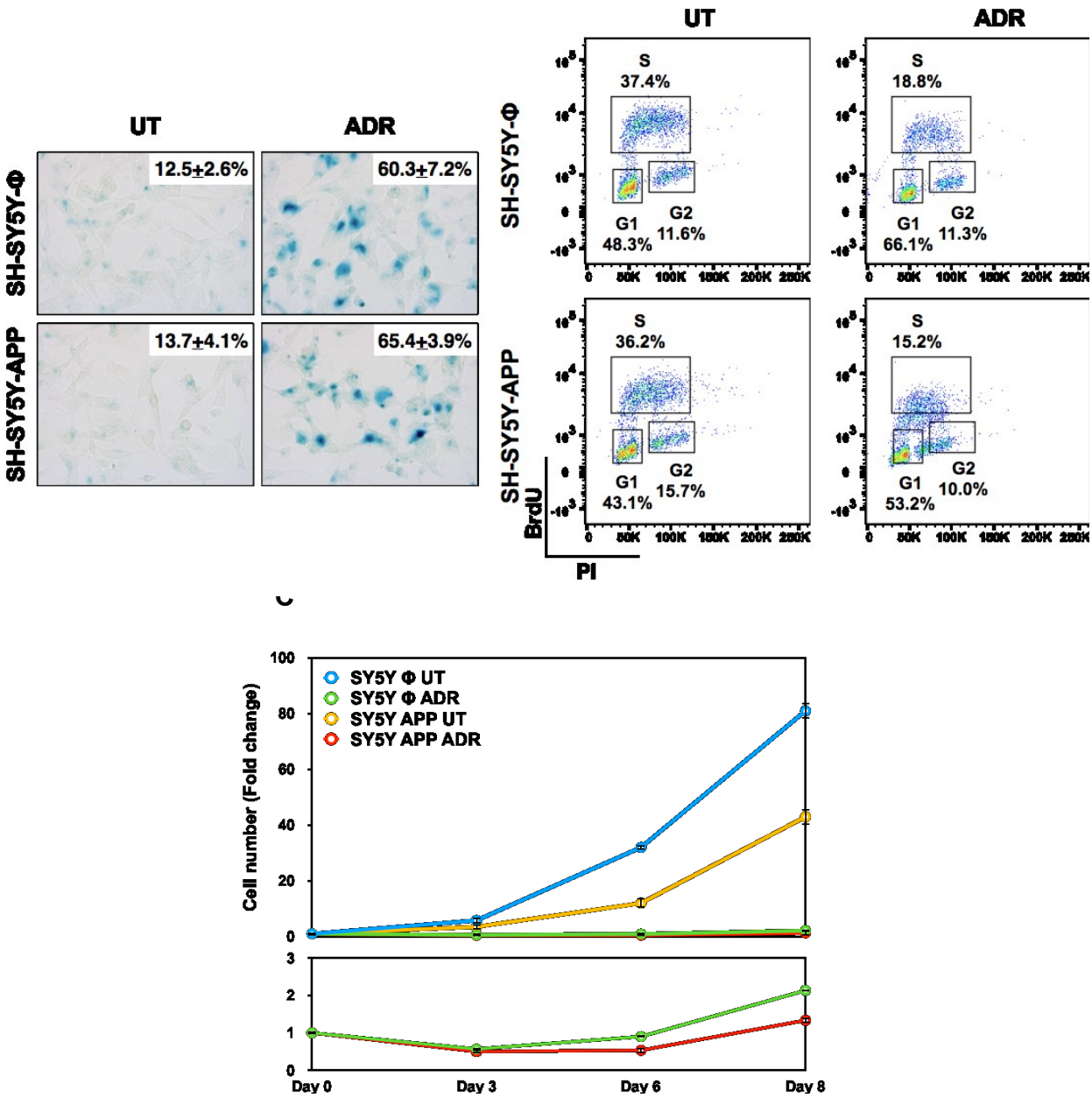


Figure 15: Inducing TIS in neuroblastoma cell line SH-SY5Y. (A) A subset of the cell population turned blue via SA-β-gal assay. (B) BrdU/PI staining displayed that some cells are still in the S phase of the cell cycle. (C) Growth curve analysis of the cells (n=3) as in (A). The lower box displays the change in viability of the ADR treated cell lines with a smaller scale for y-axis for a closer look.

5 *APP* and *PSEN1* in senescence

5.1 *APP* and *PSEN1* depletion does not impact the senescing ability of a cell

To better understand the role of the underlying molecular mechanisms of AD genes in the context of senescence, knockdown loss of function approach was used. *APP* and *PSEN1* were selected as targets because these are two of the most commonly altered genes in the case of EOAD.

A construct expressing small hairpin RNAs (shRNA) against these genes was introduced in the control lymphomas to induce their knockdowns. Five sequences of shRNAs for each of the genes were tested for their ability to knockdown gene expression, monitored by real-time PCR (data not shown). The shRNAs that demonstrated the most significant knockdown levels were therefore selected for further experiments. Sufficient levels of knockdown were obtained for both gene players. *APP* and *PSEN1* expression levels were knocked down to less than 30% and 1% of endogenous levels, respectively (Figure 16A).

These APP and Psen1 depleted cells were then treated with ADR to induce TIS. SA-β-gal and BrdU/PI staining confirmed marginally reduced senescence (Figure 16B) that did not achieve statistical significance. Thus, we can conclude that *APP* and *PSEN1* do not influence the cell's ability to undergo TIS.

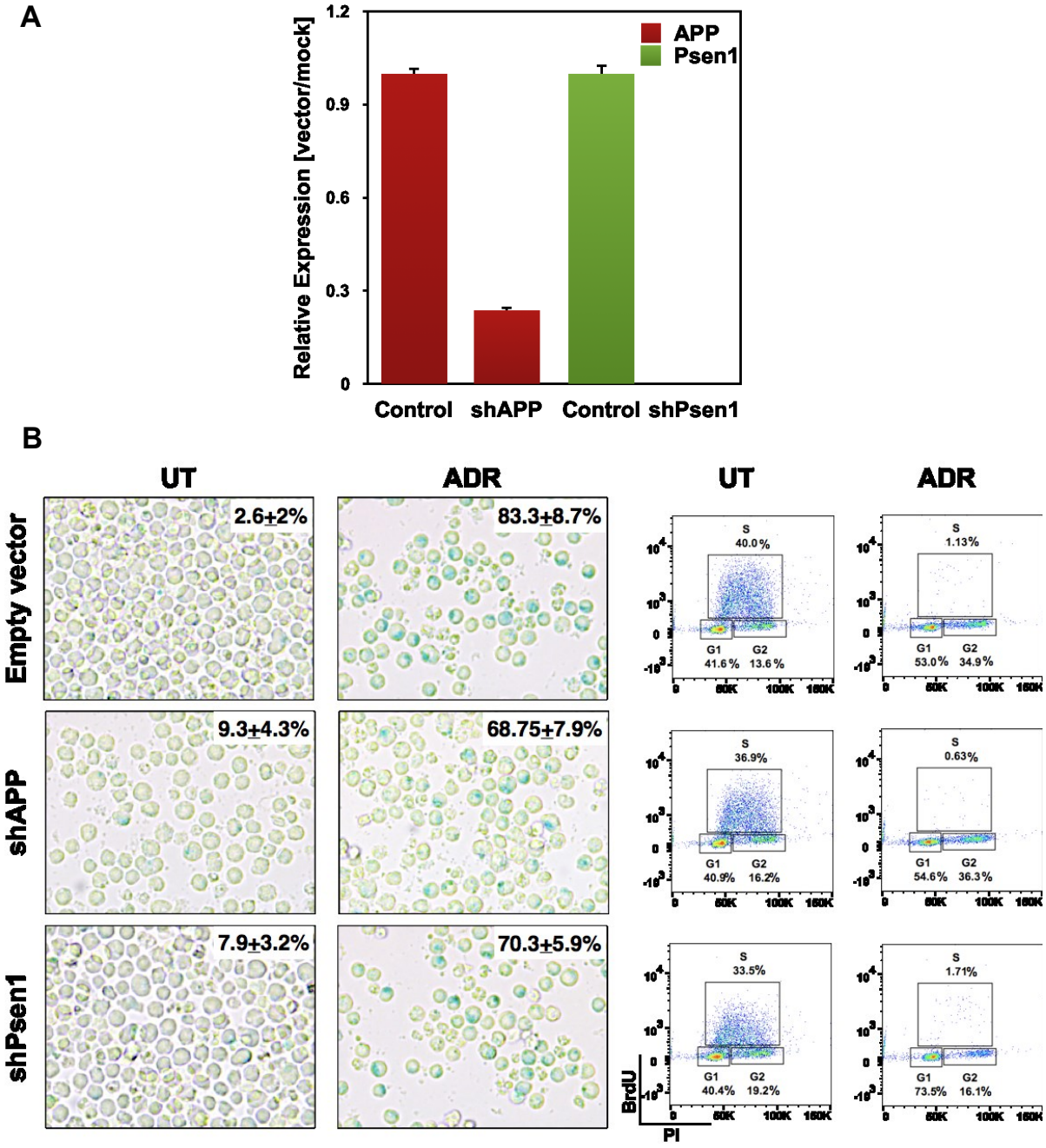


Figure 16: Senescence induction independent of APP and Psen1. (A) *APP* and *PSEN1*, most relevant players of Alzheimer's disease, were knocked down in control;bcl2 lymphomas (n=3) using shRNAs as shown here with their respective mRNA expression levels. (B) Knockdown of *APP* and *PSEN1* did not affect the ability of the cells to undergo senescence. This was preliminarily confirmed by SA-β-gal (left), as well as by BrdU/PI staining (right).

5.2 APP depletion reduces ER stress associated with senescence

In the context of neuroblastoma cell lines, APP overexpression induced ER stress and autophagic lysosomal burden as shown in Figure 14. This suggests for a role of APP in these processes. To determine whether APP contributes to senescence associated ER stress and the autophagic-lysosomal burden in control;bcl2 lymphomas, along with other senescence features, knockdown loss of function approach was used.

As observed in Figure 16B, both the control and shAPP-transduced lymphomas exhibited TIS. Subsequently, their expression patterns were compared by immunoblotting. A reduced protein expression level of APP was observed (Figure 17) in the untreated shAPP-transduced lymphomas. But, after TIS induction, the expression of APP was similarly up regulated as in the control-transduced lymphomas.

Importantly, a dynamic pattern of TIS associated expression of various proteins was observed (Figure 17). The expression level of p21 was up regulated in the untreated shAPP-transduced lymphomas, whereas H3K9me3 expression was down regulated, as compared to the untreated control-transduced lymphomas. However, p21 expression levels were further up regulated in shAPP-transduced lymphomas post TIS. On the other hand, the H3K9me3 levels were up regulated similar to the control-transduced lymphomas, post TIS. Phospho-p53 expression appeared unaffected in these lymphomas.

In terms of ER-stress, there was a decrease in p62 expression when APP was knocked down. Post TIS, senescence related ER stress remained significantly lower, thus confirming the contribution of APP towards this phenomenon (Figure 17). The autophagic-lysosomal burden, detected by the expression levels of MAP1-LC3-II-isoform, was also reduced after a knocked down APP expression. However, the conversion of MAP1-LC3-I-isoform (depicted by the upper LC3 band) to MAP1-LC3-II-isoform (depicted by the lower LC3 band) was restored to similar levels post-TIS as compared to the control-transduced lymphomas. This could also be due to the incomplete knockdown of APP, which suggests that even low levels of APP are sufficient to induce autophagic burden. Alternatively, SASP and other senescence related factors could also be responsible for most of the burden on the autophagic-

lysosomal machinery. Absolute conclusions cannot be made, as there was an incomplete knockdown of APP. Moreover, post-TIS, the APP expression levels were induced again, possibly enough to partly carry out its functions. Using an APP knockout system may lead to more conclusive answers.

This suggested that APP, albeit only in part, does support TIS and establishes TIS as a complex process involving concerted regulation by several proteins which need to be further studied.

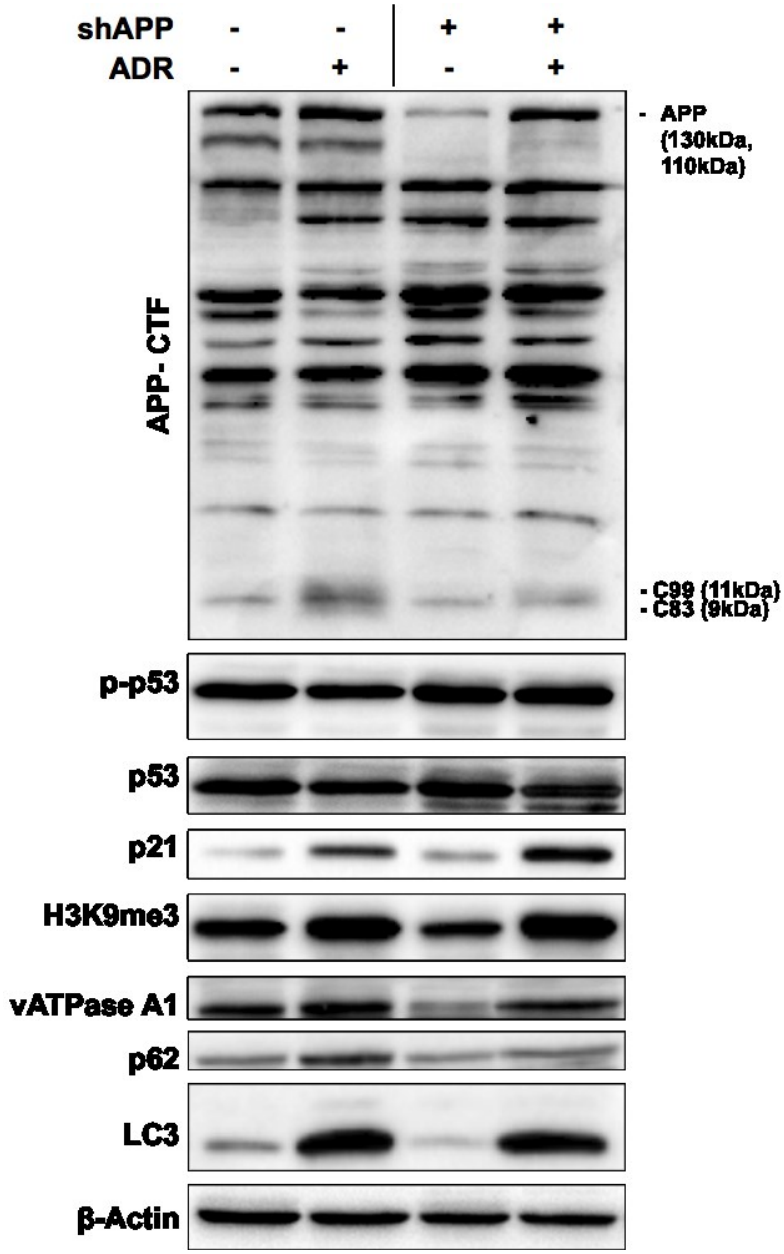


Figure 17: Correlating APP with senescence markers. This immunoblot demonstrates the expression of APP in untreated and TIS, control-transduced lymphomas (n=3) and shAPP-transduced lymphomas. The correlation between APP and the expression of markers of senescence was determined by comparing their protein expression patterns. The impact of knocking down APP on ER-stress and autophagic-lysosomal burden was depicted by analyzing the expression levels of markers such as p62, MAP1-LC3-I-isoform (upper band) to MAP1-LC3-II-isoform (lower band) conversion. Elevated autophagic flux, and activation of the lysosomal vacuolar type H^+-ATPase was observed by vATPase A1 levels. The expressions of all the proteins are normalized using β-actin.

6 Inhibition of APP processing using a Gamma-secretase inhibitor

We observed that APP overexpression burdens the cellular system with proteotoxic stress, whereas, depleting the system of APP reduces this stress. To narrow down the mechanism by which APP strains the cells, especially in TIS, APP processing was specifically targeted. Figure 4 illustrates various enzymes that are responsible for cleaving APP into smaller peptides.

γ-secretase, along with β-secretase or α-secretase, are key enzymes involved in the processing of APP. γ-secretase cleavage in combination with β-secretase forms the Aβ peptide and plaques- the major culprit behind the AD pathology[176–181]. Many γ-secretase inhibitors were or are in clinical trials for improving the prognosis of AD. Therefore, the first question that intrigued us was whether APP-cleavage, specifically via the γ-secretase pathway, contributes towards senescence and inflicts a proteolytic burden in a cell via its processed product, Aβ.

The γ-secretase inhibitor Semagacestat was chosen for further studies because its effectiveness on the γ-secretase inhibition has been confirmed *in vitro*[182]. As previously mentioned, although the drug had advanced to the phase III of the AD clinical trials for showing the potential to reduce Aβ levels in the plasma, it failed to reduce Aβ in the cerebrospinal fluid and brought about many other side effects[127–129].

6.1 Promising senolytic effects of Gamma-secretase inhibition

To begin with, the effect of γ-secretase inhibition on the viability of the TIS control;bcl2 lymphomas was determined. The effect of γ-secretase inhibition on the viability of the untreated, proliferating control;bcl2 lymphomas was used as a control.

TIS was induced in control;bcl2 lymphomas by ADR treatment. On day 5 after senescence induction, Semagacestat was added to inhibit γ-secretase in TIS as well as the non-treated/non-senescent control;bcl2 lymphomas, and results were analyzed after 2 days. Treating the non-senescent lymphomas with Semagacestat did not affect their viability, that is, the cells continued growing exponentially (Figure 18A). But in case of TIS, the viability of the cells was reduced post inhibition (Figure 18B). This suggests that the γ-secretase inhibitors like Semagacestat might have senolytic properties. However, the exact mechanism of these effects in senescent cells needs to be further investigated.

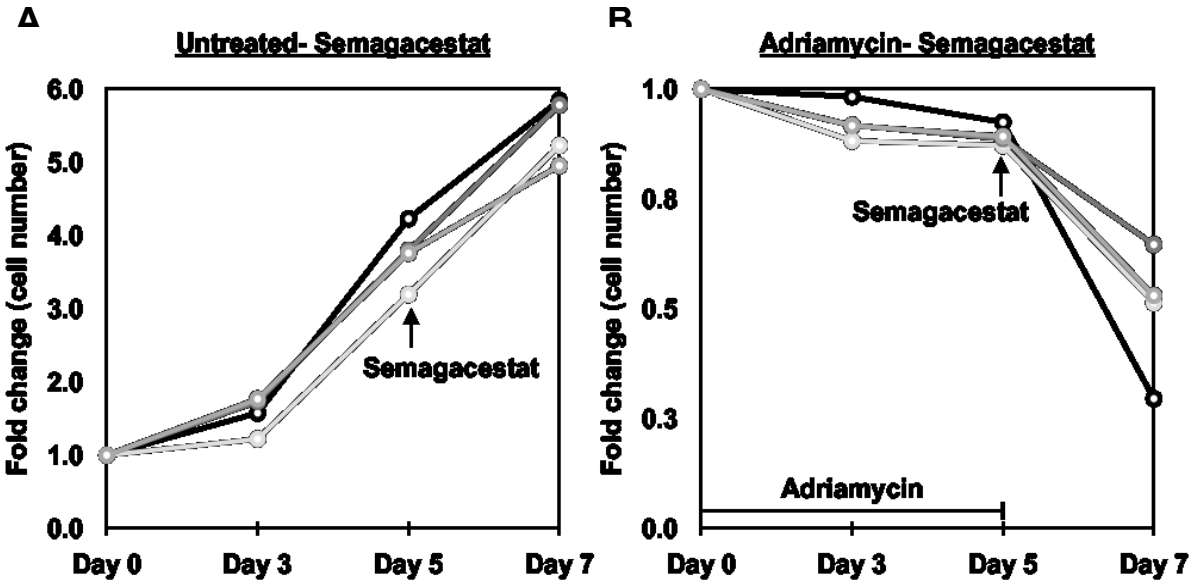


Figure 18: Senolytic effects of Semagacestat. Control lymphomas were sequentially treated- with ADR to induce senescence, followed by Semagacestat, and the growth curve was plotted to analyze the effects on viability. (A) In non-senescent control;bcl2 lymphomas, Semagacestat did not affect the viability of the cells. (B) In TIS control;bcl2 lymphomas, inhibiting γ-secretase enzyme led to expedited cell death (n=4).

6.2 Gamma-secretase inhibition does not demonstrate disruption of the APP processing in control;bcl2 lymphomas

Disruption of APP processing via γ-secretase was aimed to demonstrate the impact of restricted Aβ production in non-senescent as well as senescent lymphomas. Since knocking down APP resulted in reduction of senescence related ER stress, targeting γ-secretase enzyme was an approach to decipher the mechanism by which APP contributes to this stress.

As mentioned above, γ-secretase, depending on the combination with other enzymes (β-secretase or α-secretase) results in the formation of different peptides, such as APP intracellular domain (AICD) (~7 kDa), p3 (~2.5 kDa) and Aβ (~4.5 kDa), which are secreted in the extracellular environment or are transported to the nucleus (Figure 4). γ-secretase cleaves towards the c-terminal of APP, therefore, when γ-secretase is blocked, the CTFs remain intracellular and membrane bound. We analyzed for the expression of APP and APP-CTFs, via immunoblot analysis, using APP-CTF specific antibody. We observed that the inhibition of the γ-secretase in lymphomas did not result in an accumulation of the APP-CTFs in either UT or TIS setting (Figure 19A). Proteolytic processing of APP was also determined by immunofluorescence staining, using the same APP-CTF specific antibody. Although no significant differences were observed by immunoblot analysis, immunofluorescence staining showed more sensitivity in detecting significant changes in the cumulative expression levels of APP and its CTFs after γ-secretase inhibition (Figure 19B).

From this we can conclude that in TIS of control;bcl2 lymphomas, there is an increase in the expression levels of APP. The inhibition of its processing further led to the accumulation of its CTFs within the cell. Although the change in their expression levels was out of scope to be detected by immunoblotting, detection by immunofluorescence demonstrated an increase in cumulative accumulation of APP and APP-CTFs.

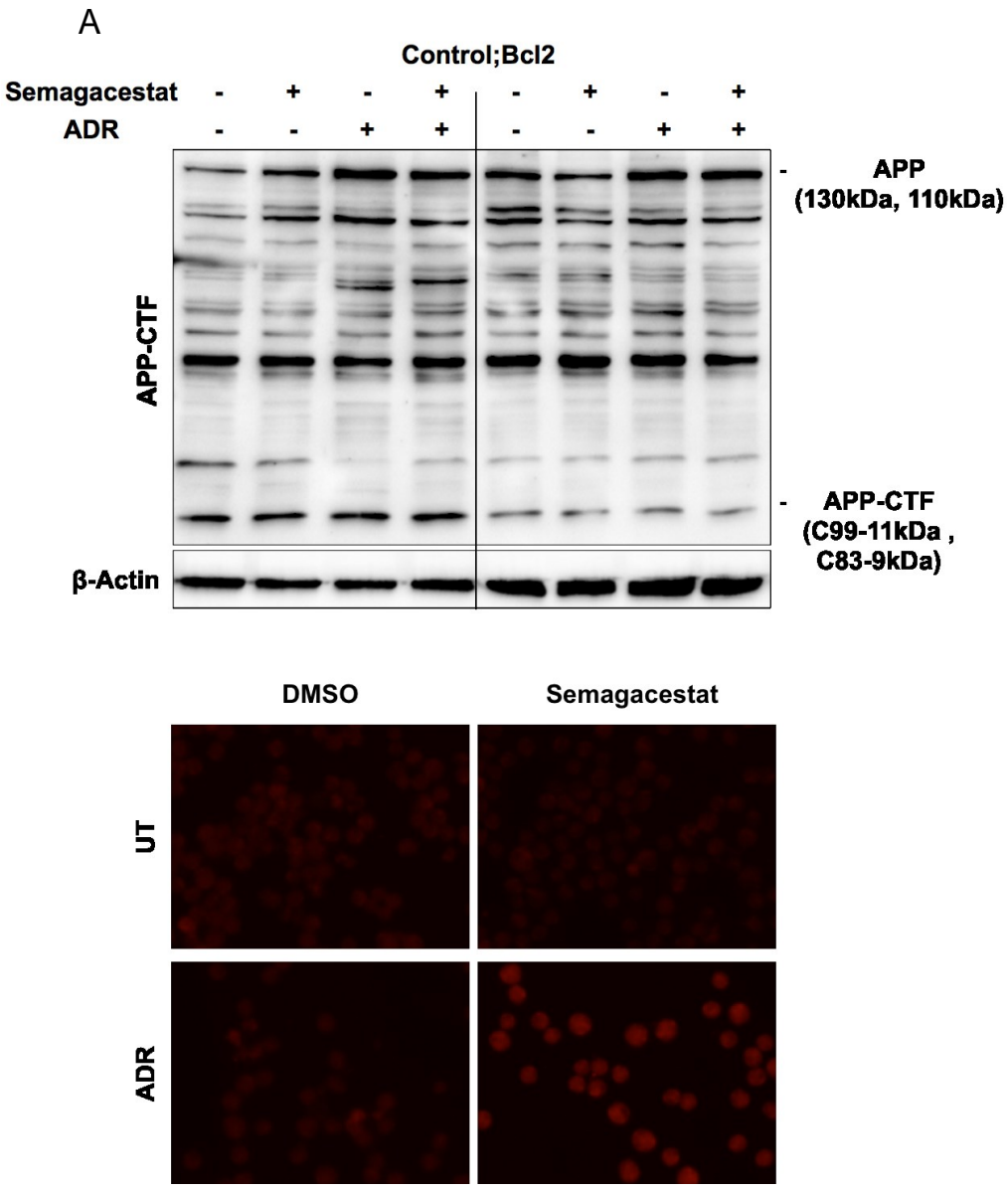


Figure 19: γ-secretase inhibition in a control;bcl2 lymphomas. (A) Cell lysates of control;bcl2 lymphoma cells (n=4) treated with ADR and Semagacestat were analyzed by immunoblot to visualize APP processing. (B) The distribution of the APP full-length protein and C-terminal peptides was observed by immunofluorescence.

6.3 Gamma-secretase inhibition impedes APP processing in the APP overexpressing neuroblastoma cell line

Since we were unable to observe a conclusive effect of Semagacestat on APP processing in control;bcl2 lymphomas by immunoblotting (Figure 19A), the experiment was duplicated in the SH-SY5Y cell lines. This was done to validate and confirm the function of Semagacestat as an inhibitor of APP processing at the selected concentration.

The SH-SY5Y cell lines, parental and APP overexpressing, were treated with Semagacestat for 2 days. It did not have any effect on the viability of the cell line in either setting (data not shown). The effect of γ-secretase inhibition on APP processing was observed by immunoblot analysis. In the case of parental SH-SY5Y cell line, we observed no significant accumulation of APP-CTFs post γ-secretase inhibition. It is possible that the levels of these peptides are below the threshold of detectability by these techniques (Figure 20A). However, in APP overexpressing SH-SY5Y cell line, there was a significant accumulation of the APP-CTFs, thus validating the inhibition of γ-secretase by Semagacestat. Additionally, the effect of γ-secretase inhibition on APP and its CTFs was also observed by immunofluorescence, using the same antibody as in the immunoblot, which is specific for the CTF of APP (Figure 20B). The antibody generates a cumulative fluorescence signal for full length APP, as well as its intracellular CTF. Like immunoblotting, the effect of γ-secretase inhibition in the APP overexpressing SH-SY5Y cell line was more pronounced.

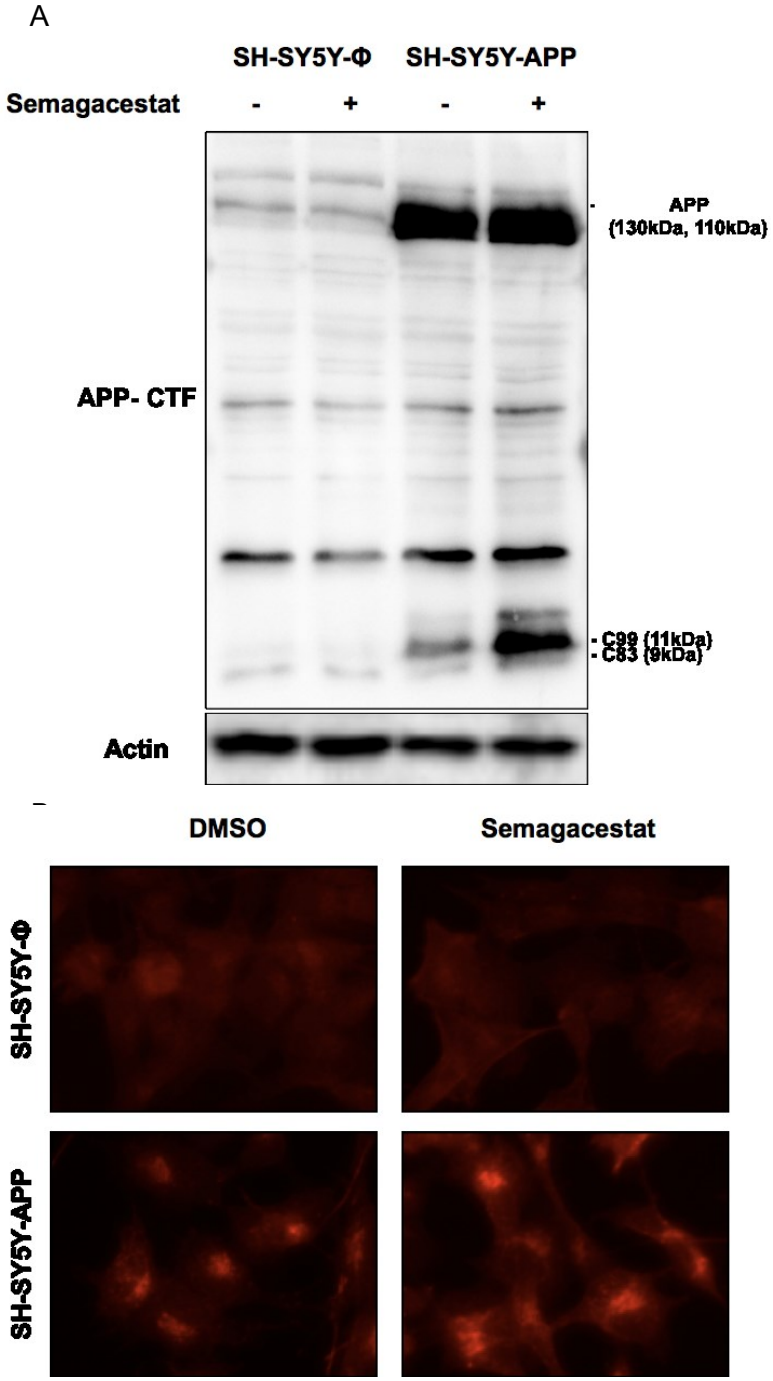


Figure 20: γ -secretase inhibition in a Neuroblastoma cell line. (A) The accumulation of APP CTFs were observed only in case of APP overexpressing SH-SY5Y cell line (n=3), by immunoblotting. The parental cell lines did not demonstrate an accumulation of the CTFs. (B) The distribution of the proteins and peptides were observed by immunofluorescence, which showed similar results as the immunoblot, for APP and its intracellular CTFs.

Hence, we could confirm the inhibition of the γ-secretase enzyme, which further impedes the final cleavage of APP and leads to the accumulation of its CTFs. Although γ-secretase was inhibited in both the parental as well as the APP over expressing SH-SY5Y cells, its effects were visible in case of APP over expressing cell line only. This suggests a possibility that the endogenous expression levels of APP are critical for visualization by routine techniques. This could also be an explanation why the effects of γ-secretase inhibition was not visible in control;bcl2 lymphomas.

6.4 Gamma-secretase inhibition in the control;bcl2 lymphomas does not affect TIS associated proteotoxicity nor other senescence markers

Although no effect of γ-secretase inhibition on APP processing was observed in the control;bcl2 lymphomas, we further pursued to understand its effect on other markers of senescence and proteotoxicity. This was done because we observed a senolytic potential of Semagacestat in control;bcl2 lymphomas which suggested some effect by some unknown mechanisms. Moreover, the inability to confirm the effect of the drug on APP processing by immunoblotting, possibly due to low endogenous APP levels, could be a drawback of the model system.

After γ-secretase inhibition, the senescent cells maintained a positive SA-β-gal staining, with a slight decrease in the percentage of senescent cells (Figure 21A). Immunoblot analysis revealed that the inhibition of γ-secretase did not affect the observed markers of senescence or proteotoxicity (Figure 21B). Therefore, in conclusion, blocking the processing of APP via the γ-secretase enzyme in control;bcl2 lymphomas did not have an impact on TIS. Moreover, the APP products formed by γ-secretase cleavage do not significantly contribute to proteotoxic stress associated with TIS. This implies that proteotoxic stress exhibited in TIS by APP is Aβ-independent.

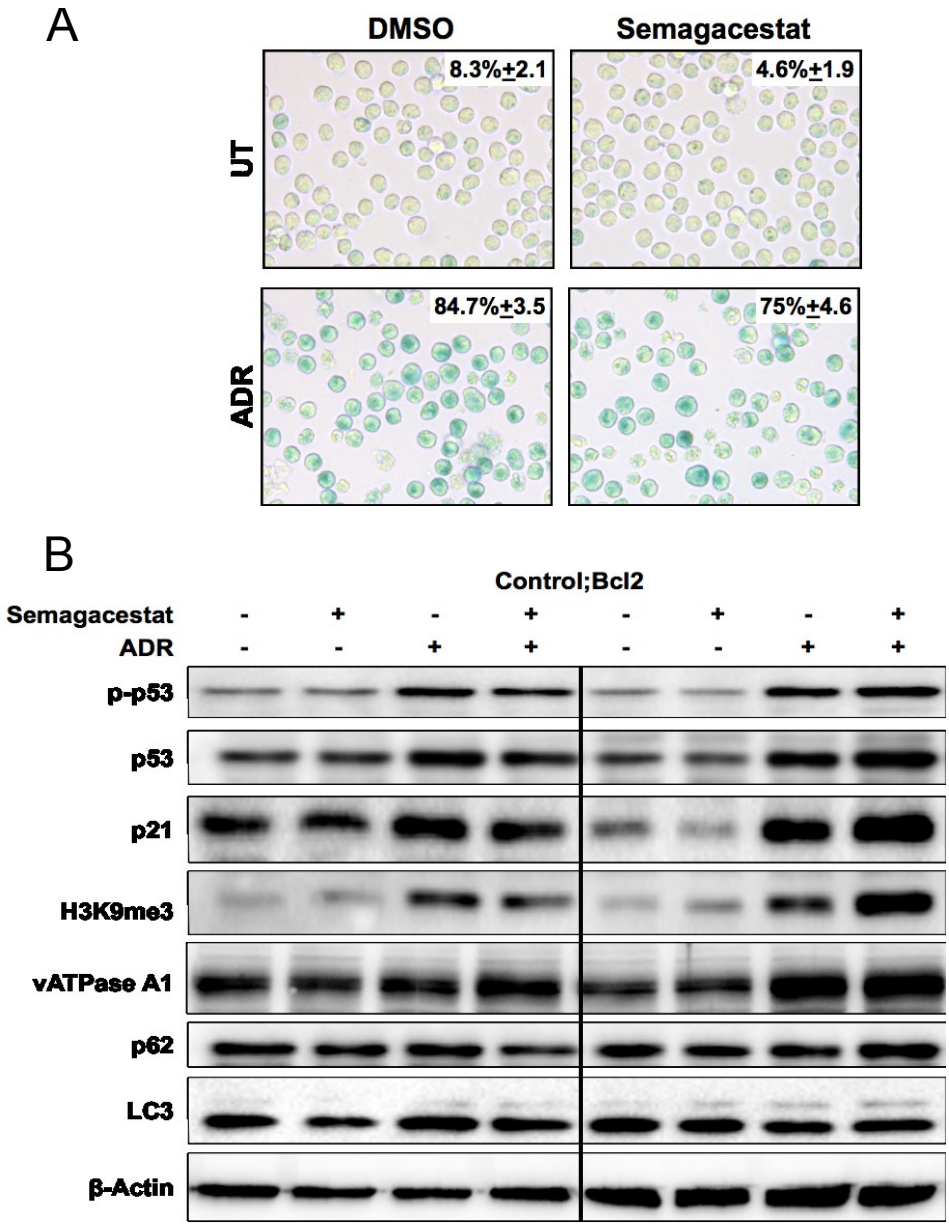


Figure 21: Mechanistic implications of γ-secretase inhibiton on senescence markers in control;bcl2 lymphomas. (n=4) (A) Effect of γ-secretase inhibition of senescent cells was observed preliminarily by SA-β-gal assay. (B) Immunoblot analysis indicating the effect on markers of senescence when gamma-secretase was inhibited. β-actin was used as a loading control.

6.5 Gamma-secretase inhibition in the neuroblastoma cell line marginally affects proteotoxicity as well as other senescence markers

Subsequently, the next step was to investigate whether blocking the cleavage of APP via γ-secretase inhibition in SH-SY5Y cells, would impact the expression of senescence and proteotoxicity markers. Since APP overexpression induced these stresses in the cell line, we were interested to know if blocking its processing would alleviate them.

The expression of majority of the senescence markers appeared to be unaffected when the γ-secretase enzyme was inhibited in case of the SH-SY5Y parental as well as APP overexpressing cell line (Figure 22). There was a slight decrease in the expression of p21 and MAP1-LC3-II isoform post γ-secretase inhibition in both settings. This suggests inhibiting the γ-secretase marginally decreased that autophagic-lysosomal burden, possibly caused by the processing of APP. This also reiterates the observation from knocking down APP in control;bcl2 lymphomas (Figure 17), suggesting APP partly contributes to the autophagic-lysosomal burden via γ-secretase mediated processing. On the other hand, pattern of p21 expression cannot be explained. This is because knocking down APP increased p21 expression in control;bcl2 lymphomas but inhibiting γ-secretase led to a decrease in its expression both parental and APP overexpressing SH-SY5Y cell lines.

In conclusion, it could be predicted that Aβ and other by-products of γ-secretase cleavage do not exclusively account for the role of APP in proteotoxicity. Since knocking down APP significantly reduced ER stress as well as autophagic-lysosomal burden (Figure 17), there could be other pathways or mechanisms via which APP affects these processes.

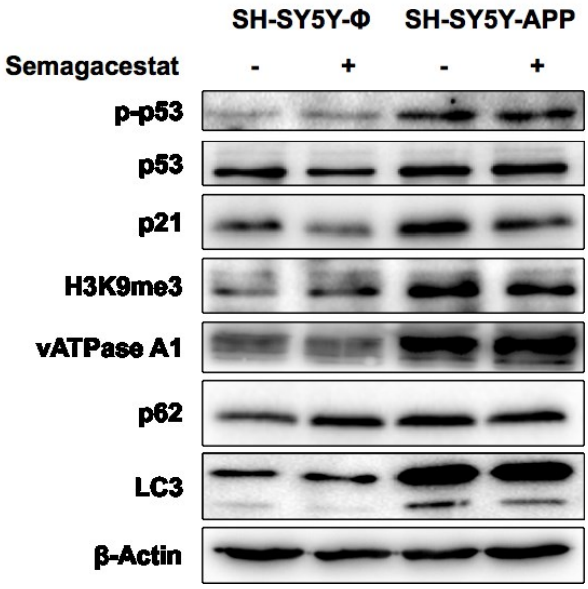


Figure 22: Mechanistic implications of γ-secretase inhibiton on senescence markers in neuroblastoma cell line. Immunoblot analysis indicating the effect of γ-secretase inhibition on markers of senescence and proteotoxicity, which were influenced by the overexpression of APP in SH-SY5Y cell lines (n=3), β-actin was used as the loading control.

DISCUSSION

Proteostasis is an essential mechanism in a cell, necessary to keep up with the continuous protein turnover throughout its development. Undesirable or excess proteins in a cell have an adverse effect on its homeostasis. Cells that experience stresses such as ROS, radiation, etc. thereby initiating DDR, could also incur the accompanied protein damage. Misfolded, unfolded or erroneous proteins could cause proteotoxicity. Hence, strict regulation of the proteostatic machinery is highly important[159].

Proteotoxicity has long been associated with neurodegenerative disorders. From a histological perspective, the misfolded and/or aggregated proteins are present in the affected areas of the brain and have thus been established as the pathological hallmarks of their respective neurodegenerative disorders. Moreover, genes that are associated with an increase in the instances of their respective protein aggregation have been linked to the familial forms of the neurodegenerative disease. Understanding the role of these genes and the impact of their mutations on the aggregation of the proteins improves our understanding of the disease pathology[183]. For e.g., missense mutations in APP engender accumulation of Aβ in AD. Transgenic mouse models that over express the disease associated mutant human genes result in the aggregation of their associated proteins. This results in some of the clinical and pathological symptoms of the disease[184].

On the other hand, our lab has recently established that there is proteotoxic stress coupled with SASP, which leads to senescence-associated metabolic reprogramming. SASP factors disrupt the protein homeostasis in a cell due to an increase in production of cytokines and chemokines. Senescent cells have increased senescence related oxidative stresses that elevates the formation of misfolded proteins. This leads to ER stress, UPR and autophagic-lysosomal burden, correlated with senescent cells[70].

1 Therapy-induced cancer cell senescence, SASP and Alzheimer Disease

Identification of genes that are involved in AD has been complicated. Genome-Wide Association Studies (GWAS) and next-generation sequencing (NGS) studies have unveiled that a lot of genes that appear to be involved in AD are also linked to several other pathways, such as lipid metabolism, immune response, and endocytosis/synaptic function[185]. Some of these genes have been curated and presented in the KEGG pathway for AD. A subset of these genes appeared to be altered in TIS control;bcl2 lymphomas. But on the other hand, in other types of senescence such as OIS of MEFs, the up regulation of the AD machinery was not observed. It could be due to different mechanisms behind OIS in MEFs and TIS in murine control lymphomas[186]. Therefore, in this thesis, we discuss the crosstalk between therapy-induced cancer cell senescence and AD.

We observed that in control;bcl2 lymphomas, TIS affected the expression levels of AD genes. However, in senescence incapable Suv39h1$^{-/-}$;bcl2 lymphomas these genes were minimally altered after ADR therapy. This data showed that the AD machinery is significantly more pronounced in TIS. Moreover, the alteration of the AD machinery was not recapitulated when senescence was induced independent of a SASP response. SASP and SASP related proteotoxic stress is complimentary with ADR induced TIS but not with rapamycin-induced senescence[70] (Figure 23). This suggests that although the role of AD machinery is correlated with TIS in control;bcl2 lymphomas, it is only observed when SASP is also present. Likewise, suppression of SASP by NF-κB-inactivation resulted in lower expression levels of the AD machinery. This reinforced our rationale that the AD machinery is correlated to TIS, via SASP.

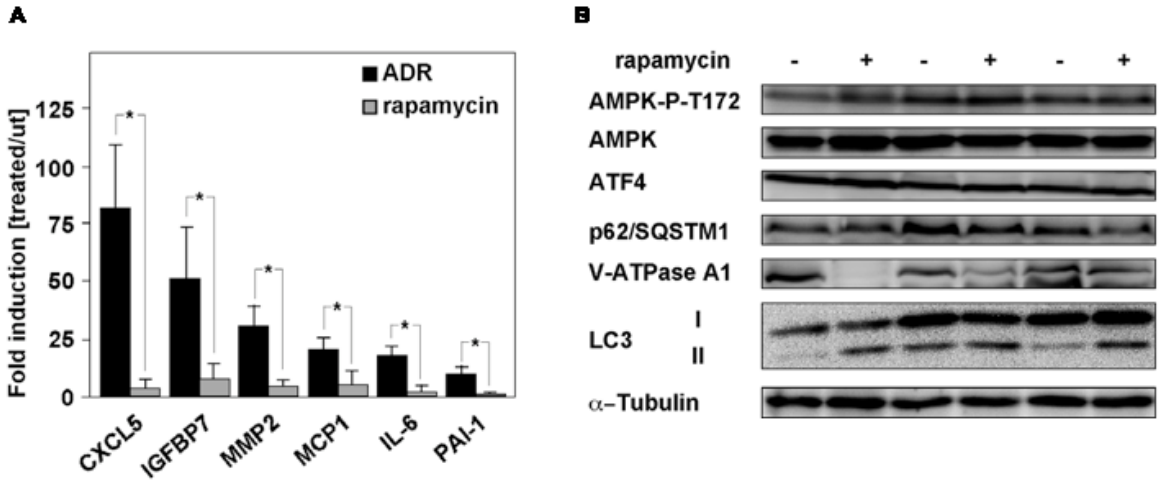


Figure 23: Rapamycin-induced senescence, SASP and proteotoxicity. Dörr et al depicted that ADR induced TIS but not rapamycin induced TIS of control;bcl2 lymphomas presents with SASP and its related proteotoxic stress. (A) RQ-PCR demonstrated the difference in the gene expression levels of SASP factors in rapamycin treated/untreated lymphomas as compared to ADR treated/untreated. (B) They further demonstrated that rapamycin-induced senescence does not accompany energy-demanding proteotoxicity-driven ER stress, although the autophagic machinery is elevated. This was shown by immunoblot analysis of AMPK-P-T172, AMPK, ATF4, p62/SQSTM1, V-ATPase A1 and MAP1-LC3-I/II of untreated *vs.* rapamycin induced TIS control;bcl2 lymphomas.

Additionally, Sheng and colleagues observed that LPS induced neuroinflammation in the brain of APP transgenic mice upregulated APP as well as its cleavage via the amyloidogenic pathway[187]. However, in control;bcl2 lymphomas, LPS induced inflammatory response did not affect the expression of the two major AD genes-APP and Psen1. This confirmed that in control;bcl2 lymphomas both TIS and its associated inflammatory response (SASP) are essential for the induction of the AD machinery.

2 APP and Psen1 may contribute to AD via senescence

It is widely known that senescence is a concurrent companion of age-related pathologies[132]. Accumulation of senescent cells in the affected tissues is associated with the onset of the disease. In an AD inflicted brain, cells such as astrocytes and microglia display features of senescence[188]. Corresponding to this, we found that particularly in the brain of older *APPPS1*$^{+/-}$ transgenic mice, senescent cells were present in abundance. This implies that when genes related to AD are altered in these transgenic mice, it not only results in the development of Aβ plaques but also causes more brain cells to senesce. By directly correlating the SA-β-gal positive cells with the Aβ plaques, we realized the simultaneous presence of senescent cells and features of AD in these mice.

From a senescence view point, we observed an upregulation of *APP* and other AD genes in TIS control;bcl2 lymphomas. Similarly, when TIS was induced in control;bcl2 lymph node bearing mice by CTX treatment, the associated AD genes should be upregulated. Therefore, in line with the transgenic APPPS1$^{+/-}$ mouse model, where we postulate that senescence precedes the formation of Aβ plaques, we analyzed the lymph node sections comprising of control;bcl2 lymphomas. We observed for the presence of plaques in TIS control;bcl2 lymph nodes. We were unable to detect Aβ plaques in the TIS lymph nodes sections. It could be because formation of plaques might be a brain specific phenotype. Alternatively, one could postulate that even if Aβ was expelled from the lymphoma cells, the flow through of fluids via the lymph nodes could wash away the secreted or exocytosed products.

3 Senescence related stress on autophagic-lysosomal machinery can potentially contribute to AD pathology

The link between a faulty lysosomal network and the mechanism involved in neurodegeneration is well studied[189–192]. A neuron is a differentiated cell that is unable to dilute the toxic protein buildup by cell division. Therefore, the efficiency of the lysosomal machinery is fundamental for their survival. The inability to achieve this results in the accumulation of aggregated proteins and peptide fragments, which induce toxicity in the cell[193]. Certain autophagy-related genes along with defective autophagic-lysosomal machinery have also been correlated with neurodegenerative diseases. The risk factors driving the formation of Aβ peptides and other AD pathology also influence the lysosomal network. Amelioration of the lysosomal network dysfunction has been observed to have therapeutic effects on the pathological and cognitive deficits in animal models of AD. This proves that malfunctioning of the autophagic-lysosomal machinery contributes significantly to the pathology of AD and other neurodegenerative disorders[194].

In the brains of APPPS1$^{+/-}$ transgenic mice, we found that the senescent cells appeared in young AD mice before the plaques were formed. Furthermore, Huang Yu and colleagues also demonstrated that autophagic vacuoles (AV) appear in neurons of these mice at a younger age, before Aβ plaques are formed[93]. This suggests that these gene mutations initially induce senescence as well as proteotoxicity, which are also correlated with each other. Moreover, this leads to a burden on the autophagic-lysosomal machinery, which could contribute towards some of the pathological features of an AD brain, such as plaque formation. Therefore, we postulate that these mutations of AD results in a compromised autophagic-lysosomal machinery via senescence and proteotoxicity.

Moreover, in transgenic mouse models of AD, specifically involving APP mutations, there is an accumulation of autophagy substrates like MAP1-LC3-II as well as p62 in the AV[175]. The inability to eliminate the autophagy substrates affects the clearance of many AD related proteins, such as Aβ, tau and other factors promoting cell death[195]. APP substrates and secretases are present in abundance in the AVs, where the Aβ peptides are generated during autophagy[93]. Under normal conditions, these peptides

are degraded by the lysosomes[196–198]. But in case of a compromised autophagic-lysosomal system, there is an accumulation of AVs in the cells. The Aβ produced in the endosomes is transferred to the lysosomes directly or during autophagy. These Aβ containing lysosomal compartments could be the reservoirs of Aβ immunoreactivity in AD brain[93,199]. In consensus of this, we observed that over expression of the Swedish mutation of APP, in a neuroblastoma cell line, led to an increased burden on the proteosomal and autophagic machinery, as well as the markers of senescence. APP is central to the formation of plaques. Therefore, this suggests the possibility that both these processes, induced by APP, collectively contribute to the AD pathology.

The other AD related gene, PSEN, is also known to be involved in the proteolytic machinery of a cell. It is necessary for the proper functioning of the autophagic-lysosomal system[200]. In cases where PSEN1 mutations contribute to AD, a disruption in the lysosomal proteolysis as well as autophagy is observed[92]. Therefore, it can be postulated that in the transgenic AD mouse models, PSEN mutation also contributes towards the disruption of proteostasis, leading to proteotoxicity, which precedes Aβ plaque formation in an AD inflicted brain[93]. Since all these features also overlap with features of a senescent cell, one can hypothesize that these genes could also be involved in senescence.

4 APP overexpression by itself does not induce TIS in a neuroblastoma cell line

Apart from the control lymphomas, TIS is also relevant with respect to some cancer cell lines. In case of neuroblastoma, we observed that APP overexpression in SH-SY5Y cell line led to the up regulation of the markers of senescence, including markers of autophagic-lysosomal burden and ER stress, suggesting an induction of a pre-existing senescence burden. This also indicated a mechanistic correlation between APP and senescence. Although the proteotoxicity burden could be the reason for the slower growth rate of APP overexpressing SH-SY5Y cells compared to the parental cells, it was not enough to induce senescence. This could be due to the robustness of the immortal cell line. Moreover, despite the pre-existing senescence burden by APP in the SH-SY5Y cell line, there was no influence on its response to ADR induced TIS, as compared to the parental cell line.

Additionally, both the parental as well as the APP overexpressing neuroblastoma cell lines displayed many features of senescence; but only a subpopulation of the cells were senescent after ADR treatment. This could be due to the possibility that there are some other factors involved, which prevented the remaining cells from undergoing senescence, such as suboptimal levels of ADR dosage. Further work in additional cell lines, as an alternative model system, would be required to confirm the association between APP overexpression and TIS.

5 APP contributes to senescence associated ER stress

It has been well studied that APP, PSEN1, and their mutations contribute towards proteotoxicity in a cell in many different ways[92,175,200]. It could be either by over burdening of the proteosomal system or incapacitating the autophagic machinery or both. These systems are extremely important for maintaining autophagy balance in a senescent cell[70]. An imbalance in these systems could result in disturbing the senescent state of the cell. When we induced TIS in *APP*, and *PSEN1* knockdown control;bcl2 lymphomas, there was no difference in their senescing ability as compared to the controls. This suggested that although there is an overlap between features of senescence and AD related genes in a cell, they are a part of a complicated biological process. It is possible that the impact of these players is one

of the many regulatory factors involved in senescence. Therefore, just altering these genes did not culminate into a significant effect on senescence.

Looking closely, knocking down APP expression by shAPP-transduction also led to the reduction of its protein levels. However, post TIS its expression was restored as in the TIS control-transduced lymphomas. This implies that the protein expression of APP in lymphomas is closely regulated and over a certain limit the APP protein is possibly degraded. Furthermore, the opposite impact of reduced APP expression on p21 and H3K9me3 expression in ADR untreated cells does not explain its definitive mechanism in senescence. But knocking down APP expression significantly led to the mitigation of ER stress that was not restored post-TIS. This suggests that APP does contribute to ER stress associated with TIS in lymphomas. Decreasing the APP levels also reduced the autophagic-lysosomal burden in untreated cells, observed by reduced MAP1-LC3-I-isoform to MAP1-LC3-II-isoform conversion, but it was also restored to the TIS level of the controls post TIS. vATPase A1 expression was also significantly decreased when APP expression was reduced, suggesting an impact of APP on autophagic flux and activation of the lysosomal vacuolar type H^+-ATPase. But since the autophagy is restored to the control state post-TIS, the expression of vATPase A1 is also restored.

6 Inhibition of APP processing using a Gamma-secretase inhibitor

γ–secretase is a lucrative target for AD. Its inhibition causes interception in the formation of Aβ, the key culprit of the disease[201]. Theoretically, inhibiting this enzyme should alleviate the influence of Aβ towards the autophagic and proteotoxic stress caused by the accumulation of these peptides[202–206]. We used SH-SY5Y cell line overexpressing APP to determine the effects of γ–secretase inhibition and to understand the probable link between senescence and APP. These cells were chosen because they are commonly used as a model system to study AD[207]. Moreover, an APP overexpression helped in better visualization of the substrate since endogenous levels of APP are difficult to observe by many routine techniques, as was also observed by Sheng et. al.[187]. Hence, overexpression of APP is a useful tool for understanding the APP biology.

Semagacestat is one of the many γ–secretase inhibitors available and is well documented for blocking the generation of Aβ. It was selected for the γ–secretase Alzheimer Trial. However, at phase II of the trials, there was no significant impact reported on the levels of Aβ in the cerebrospinal fluid (CSF) of humans[208]. This could be a probable reason for their failure at the advance stages of the trial[209]. Interestingly, after γ–secretase inhibition in control lymphomas, we observed a decrease in the viability of senescent cells, which was not observed when solely the vehicle was administered. Moreover, γ–secretase inhibition did not appear to have any impact on the viability of the non-senescent cells. This suggests that γ–secretase inhibition can lead to the selective killing of senescent cells.

Mechanistically, γ–secretase inhibition blocks the final cleavage of the c-terminal fragment of APP and renders it membrane bound[99]. Since Aβ is not produced, we were interested in studying its impact on proteotoxicity as well as other markers of senescence. Reduction in Aβ production, in theory, should reduce proteotoxic stress in the cell due to its proteotoxic effects. APP is processed via a combination of enzymes including γ–secretase, and Aβ is considered pathologically relevant for AD. We could validate that inhibition of APP processing by Semagacestat resulted in the accumulation of C99 and C83 fragments in SH-SY5Y cell line overexpressing APP, thus confirming the blockage of Aβ production. Immunoblot analysis confirmed the size of the accumulated peptide, whereas immunofluorescence confirmed increase in signal pertaining to cumulative APP and its membrane bound C-terminal fragments. However, this did not convincingly reduce the expression of senescence or the proteotoxicity markers induced by overexpressing APP. This suggests that although APP contributes towards the senescence and the proteotoxic features in a cell, inhibiting γ–secretase to impede Aβ production is not enough to rescue the cells from these effects. It is also possible that Aβ is a contributing factor towards these phenotypes but it is not the exclusive factor. There could be other APP processing products or their downstream effectors involved that may have the same or greater effect on these phenotypes.

In control lymphomas undergoing TIS, the γ–secretase inhibition did not result in an accumulation of the C-terminal fragment, as observed by immunoblot analysis. Nonetheless, some increase in signal was observed by immunofluorescence. This

could be due to the previous reports and observations that endogenous expression levels of APP might be lower than the detection threshold of immunoblot analysis. But since senescence leads to an upregulation of APP levels, blocking APP processing resulted in further accumulation of APP and its fragments. Furthermore, senescent cells have a stressed proteosomal as well as autophagic-lysosomal machinery, and inhibiting γ–secretase might add onto the backlog for clearance via the aforementioned mechanisms. Additionally, similar to our observation in neuroblastoma cell lines, expression of senescence and proteotoxicity markers remained unchanged post γ–secretase inhibition in UT as well as TIS control;bcl2 lymphomas.

Knocking down of APP in control;bcl2 lymphomas resulted in a reduction in senescence associated ER stress, whereas inhibiting Aβ production via γ–secretase did not alleviate the ER stress. Therefore, we conclude that APP induces ER stress through an Aβ-independent mechanism.

Interestingly, another study suggested that before the Aβ plaques appear in triple-transgenic mice (3xTgAD) overexpressing Swedish-mutated amyloid precursor protein, P310L-Tau (Tau_{P301L}), and physiological levels of M146V-presenilin-1 ($PS1_{M146V}$), it demonstrates C99 accumulation. This is considered to be an initiator of the neurodegenerative process and cognitive alterations in this model system[210]. They demonstrated that C99 accumulated due to an impaired autophagic-lysosomal machinery rather than a deteriorated γ–secretase processing. They also established that an impaired autophagic-lysosomal machinery results in the accumulation of C99, which further contributes to the dysfunction of this machinery[211]. This could also be a plausible alternative explanation of the senolytic behavior of the γ–secretase inhibitor that was observed in control lymphomas. Although the expression levels of these players were below the detection thresholds, it can be postulated that the accumulation of C99 after γ–secretase inhibition could induce excess pressure on the carefully balanced autophagic-lysosomal machinery, causing the cells to succumb to proteotoxicity.

Most of the studies of APP and other components of AD are performed in neuronal systems. Even though APP is a ubiquitous protein, their expression level varies from one cell type to the other. The APP protein and mRNA expression has been

observed in the brain, thymus, heart, muscle, lung, kidney, adipose tissue, liver, spleen, skin, and intestine[212–219]. This implies that although the relevance of APP is more pronounced in the context of AD, its wide-spread expression suggests that it has alternative functions as well[220].

7 Conclusion

The existence of common underlying biology behind various pathologies and diseases can be used to an advantage. Knowledge and understanding from one disease can be incorporated for improving the prognosis of another. The senescent cells can be a threat to an organism as they possess the ability to modify their tissue environment, for better or for worse[221]. Albeit senescence has been, for the longest time, associated with ageing and cancer, there is more work being done that has shown its relevance in many other physiological and pathophysiological processes. Senescent cells have been identified during embryogenesis as well as in neurodegenerative diseases like AD[9,222]. Clearance of these senescent cells or their modification have, in the past, delayed the onset of such senescence related pathologies[221,223]. Therefore, targeting this mechanism can contribute towards prolonging health during aging.

Up regulation of the AD genes in Eμ-*myc*-driven B-cell lymphomas was observed only when TIS presented with SASP. Key components of the AD machinery, APP and PSEN1 are associated with the most common forms of FAD. There have also been evidences suggesting their direct or indirect contribution towards proteotoxicity associated with its pathology[92,200,224]. The influence of some selected AD players on proteotoxicity associated with TIS control lymphomas were investigated with the aim to discover new drug target sites to specifically eliminate senescent cells. Drugs targeting these sites could contribute towards the development of senolytic drugs.

Knock down of APP did not have a direct impact on the ability of the cells to undergo senescence. Therefore, these major AD players did not impact senescent cells as we hypothesized. However, knocking down of APP led to the reduction of senescence-associated ER stress. In conclusion, although these AD players have been shown to contribute, directly or indirectly, towards proteotoxicity as well as senescence, but they are components of a larger regulatory network involving additional players. Moreover, inhibiting APP processing with γ–secretase inhibition did not strongly alleviate the proteotoxic stress associated with senescence in neither neuroblastoma cell line nor control lymphomas. This could either mean there are other factors apart from Aβ that also contribute to the features under review or the resulting accumulation of the C99 fragments substitute for the effects of Aβ.

The primary focus of this thesis has been to explore the link between senescence and AD. According to which we could show that APP contributes towards senescence-associated proteotoxic stress, but in an Aβ-independent manner.

8 Future directions

Although we could identify that proteotoxicity and SASP is a link between therapy-induced cancer cell senescence and AD, we could not deduce the specific role of the AD genes in senescence. We concentrated our study on APP because it is widely known to be involved in genetic forms of AD, but the functions of APP are not well understood. Therefore, further work is required to understand its role, especially in the context senescence.

8.1 Can overexpression of *APP* in lymphomas lead to senescence?

As we observed in the neuroblastoma cell lines, overexpressing APP led to an upregulation of many senescence markers, generating a senescence burden. But this was not sufficient to induce senescence in an immortal cell line. Therefore, it will be interesting to see if overexpressing APP in primary cells such as control;bcl2 lymphomas would be able to induce a similar senescence burden, and potentially induce senescence. This would confirm APP as a component of the senescence pathway, since in this system we observed its upregulation in TIS.

Alternatively, overexpression of wildtype *APP*, in some cellular systems, has been shown to induce apoptosis[225]. In other studies, mutations of *APP* causes ER stress-induced apoptosis[204]. This further suggests that inducing apoptosis via *APP* in cells overexpressing the anti-apototic gene *Bcl2* could induce senescence by avoiding apoptosis.

8.2 Elucidating the mechanism behind the senolytic effects of gamma–secretase inhibition

We observed that knocking down of APP in control;bcl2 lymphomas resulted in a mitigated ER stress even after TIS. This hints towards significant contribution of APP in causing TIS associated ER stress. Different APP-related studies suggest contrasting root causes of ER stress which could be Aβ-dependent, Aβ-independent, etc[204,225,226]. But in case of control;bcl2 lymphomas, we observed that restricting the production of Aβ by γ–secretase inhibition did not affect ER stress, suggesting that ER stress was induced in an Aβ-independent fashion. Since γ–secretase is also involved in the notch signaling pathway, it is necessary to determine the impact of notch signaling in this context[136].

Therefore, it is possible that the senolytic effects of Semagacestat could be due to C99 accumulation[211] or via notch pathway, which needs to be further investigated. Hypothetically, if Semagacestat exhibits senolytic potential via notch pathway, γ–secretase modulators can be used. γ–secretase modulators modulate γ–secretase activity instead of completely inhibiting it[227]. Therefore, by more specifically targeting APP and its processing, we can further understand its role and implications in the senescence.

REFERENCES

1. Hayflick, L. & Moorhead, P. S. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25,** 585–621 (1961).
2. Campisi, J. & D'Adda Di Fagagna, F. Cellular senescence: When bad things happen to good cells. *Nat. Rev. Mol. Cell Biol.* **8,** 729–740 (2007).
3. Serrano, M. & Blasco, M. A. Putting the stress on senescence. *Curr. Opin. Cell Biol.* **13,** 748–753 (2001).
4. Ben-Porath, I. & Weinberg, R. A. The signals and pathways activating cellular senescence. *Int. J. Biochem. Cell Biol.* **37,** 961–976 (2005).
5. Collado, M., Blasco, M. A. & Serrano, M. Cellular Senescence in Cancer and Aging. *Cell* **130,** 223–233 (2007).
6. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16(INK4a). *Cell* **88,** 593–602 (1997).
7. Kuilman, T., Michaloglou, C., Mooi, W. J. & Peeper, D. S. The essence of senescence. *Genes Dev.* **24,** 2463–2479 (2010).
8. Rodier, F. & Campisi, J. Four faces of cellular senescence. *J. Cell Biol.* **192,** 547–556 (2011).
9. Campisi, J. Aging, Cellular Senescence, and Cancer. *Annu. Rev. Physiol.* **75,** 685–705 (2013).
10. Dimri, G. P. *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci.* **92,** 9363–9367 (1995).
11. Gorgoulis, V. G. & Halazonetis, T. D. Oncogene-induced senescence: The bright and dark side of the response. *Curr. Opin. Cell Biol.* **22,** 816–827 (2010).
12. Courtois-Cox, S. *et al.* A negative feedback signaling network underlies oncogene-induced senescence. *Cancer Cell* **10,** 459–472 (2006).
13. Alimonti, A. *et al.* A novel type of cellular senescence that can be enhanced in mouse models and human tumor xenografts to suppress prostate tumorigenesis. *J. Clin. Invest.* **120,** 681–693 (2010).
14. Collado, M. & Serrano, M. Senescence in tumours: Evidence from mice and humans. *Nat. Rev. Cancer* **10,** 51–57 (2010).
15. Michaloglou, C. *et al.* BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* **436,** 720–724 (2005).
16. Braig, M. *et al.* Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* **436,** 660–665 (2005).
17. Collado, M. *et al.* Tumour biology: Senescence in premalignant tumours. *Nature* **436,** 642 (2005).
18. Chen, Z. *et al.* Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* **436,** 725–730 (2005).
19. Zhu, J., Woods, D., McMahon, M. & Bishop, J. M. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* **12,** 2997–3007 (1998).
20. Shi, Y. *et al.* Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. *Science (80-.).* **257,** 212–214 (1992).
21. Dimitroulakos, J. *et al.* Differential sensitivity of various pediatric cancers and squamous cell carcinomas to lovastatin-induced apoptosis: Therapeutic implications. *Clin. Cancer Res.* **7,** 158–167 (2001).
22. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell*

144, 646–674 (2011).
23. Muñoz-Espín, D. & Serrano, M. Cellular senescence: From physiology to pathology. *Nat. Rev. Mol. Cell Biol.* **15,** 482–496 (2014).
24. Ross, A. L., Sanchez, M. I. & Grichnik, J. M. Nevus senescence: An update. *Nevogenes. Mech. Clin. Implic. Nevus Dev.* **2011,** 117–126 (2012).
25. Fridman, A. L. & Tainsky, M. A. Critical pathways in cellular senescence and immortalization revealed by gene expression profiling. *Oncogene* **27,** 5975–5987 (2008).
26. Gonzalez, L. C., Ghadaouia, S., Martinez, A. & Rodier, F. Premature aging/senescence in cancer cells facing therapy: good or bad? *Biogerontology* **17,** 71–87 (2016).
27. Lu, T. & Finkel, T. Free radicals and senescence. *Exp. Cell Res.* **314,** 1918–1922 (2008).
28. Magda, D. *et al.* Synthesis and anticancer properties of water-soluble zinc ionophores. *Cancer Res.* **68,** 5318–5325 (2008).
29. Ewald, J. A., Peters, N., Desotelle, J. A., Hoffmann, F. M. & Jarrard, D. F. A High-Throughput Method to Identify Novel Senescence-Inducing Compounds. *J. Biomol. Screen.* **14,** 853–858 (2009).
30. Corcoran, L. M., Cory, S. & Adams, J. M. Transposition of the immunoglobulin heavy chain enhancer to the myc oncogene in a murine plasmacytoma. *Cell* **40,** 71–79 (1985).
31. Adams, J. M. *et al.* The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318,** 533–538 (1985).
32. Schmitt, C. A. *et al.* Dissecting p53 tumor suppressor functions in vivo. *Cancer Cell* **1,** 289–298 (2002).
33. Passos, J. F., Simillion, C., Hallinan, J., Wipat, A. & Von Zglinicki, T. Cellular senescence: Unravelling complexity. *Age (Omaha).* **31,** 353–363 (2009).
34. Debacq-Chainiaux, F., Boilan, E., Le Moutier, J. D., Weemaels, G. & Toussaint, O. P38MAPK in the senescence of human and murine fibroblasts. *Adv. Exp. Med. Biol.* **694,** 126–137 (2010).
35. Bartkova, J. *et al.* Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* **444,** 633–637 (2006).
36. Di Micco, R. *et al.* Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* **444,** 638–642 (2006).
37. Chicas, A. *et al.* Dissecting the Unique Role of the Retinoblastoma Tumor Suppressor during Cellular Senescence. *Cancer Cell* **17,** 376–387 (2010).
38. Kulju, K. S. & Lehman, J. M. Increased p53 Protein Associated with Aging in Human Diploid Fibroblasts. *Exp. Cell Res.* **217,** 336–345 (1995).
39. El-Deiry, W. S. *et al.* WAF1/CIP1 Is Induced in p53-mediated G1 Arrest and Apoptosis. *Cancer Res [CANCER Res.* **54,** 1169–1174 (1994).
40. Harvey, M. *et al.* In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene* **8,** 2457–67 (1993).
41. Dirac, A. M. G. & Bernards, R. Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *J. Biol. Chem.* **278,** 11731–11734 (2003).
42. Narita, M. *et al.* Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113,** 703–716 (2003).
43. Lowe, S. W. & Sherr, C. J. Tumor suppression by Ink4a-Arf: Progress and puzzles. *Curr. Opin. Genet. Dev.* **13,** 77–83 (2003).
44. Beauséjour, C. M. *et al.* Reversal of human cellular senescence: Roles of the

p53 and p16 pathways. *EMBO J.* **22,** 4212–4222 (2003).
45. Blais, A., Van Oevelen, C. J. C., Margueron, R., Acosta-Alvear, D. & Dynlacht, B. D. Retinoblastoma tumor suppressor protein-dependent methylation of histone H3 lysine 27 is associated with irreversible cell cycle exit. *J. Cell Biol.* **179,** 1399–1412 (2007).
46. Burkhart, D. L. & Sage, J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat. Rev. Cancer* **8,** 671–682 (2008).
47. Indovina, P., Marcelli, E., Casini, N., Rizzo, V. & Giordano, A. Emerging roles of RB family: New defense mechanisms against tumor progression. *J. Cell. Physiol.* **228,** 525–535 (2013).
48. Kareta, M. S. *et al.* Inhibition of pluripotency networks by the Rb tumor suppressor restricts reprogramming and tumorigenesis. *Cell Stem Cell* **16,** 39–50 (2015).
49. Zindy, F., Quelle, D. E., Roussel, M. F. & Sherr, C. J. Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* **15,** 203–211 (1997).
50. Alcorta, D. A. *et al.* Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc. Natl. Acad. Sci.* **93,** 13742–13747 (1996).
51. Palmero, I. *et al.* Accumulation of p16INK4a in mouse fibroblasts as a function of replicative senescence and not of retinoblastoma gene status. *Oncogene* **15,** 495–503 (1997).
52. Serrano, M., Hannon, G. J. & Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366,** 704–707 (1993).
53. Schmitt, C. A. *et al.* A senescence program controlled by p53 and p16INK4acontributes to the outcome of cancer therapy. *Cell* **109,** 335–346 (2002).
54. Kosar, M. *et al.* Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- And insult-dependent manner, and follow expression of p16ink4a. *Cell Cycle* **10,** 457–468 (2011).
55. Di Micco, R. *et al.* Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nat. Cell Biol.* **13,** 292–302 (2011).
56. Kennedy, A. L. *et al.* Senescent mouse cells fail to overtly regulate the HIRA histone chaperone and do not form robust Senescence Associated Heterochromatin Foci. *Cell Div.* **5,** 16 (2010).
57. Coppé, J.-P. *et al.* Senescence-Associated Secretory Phenotypes Reveal Cell-Nonautonomous Functions of Oncogenic RAS and the p53 Tumor Suppressor. *PLoS Biol.* **6,** e301 (2008).
58. Chien, Y. *et al.* Control of the senescence-associated secretory phenotype by NF-κB promotes senescence and enhances chemosensitivity. *Genes Dev.* **25,** 2125–2136 (2011).
59. Acosta, J. C. *et al.* Chemokine Signaling via the CXCR2 Receptor Reinforces Senescence. *Cell* **133,** 1006–1018 (2008).
60. Kuilman, T. *et al.* Oncogene-Induced Senescence Relayed by an Interleukin-Dependent Inflammatory Network. *Cell* **133,** 1019–1031 (2008).
61. Demaria, M. *et al.* An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev. Cell* **31,** 722–733 (2014).
62. Acosta, J. C. *et al.* A complex secretory program orchestrated by the

inflammasome controls paracrine senescence. *Nat. Cell Biol.* **15,** 978–990 (2013).
63. Xue, W. *et al.* Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* **445,** 656–660 (2007).
64. Lujambio, A. *et al.* Non-cell-autonomous tumor suppression by p53. *Cell* **153,** 449–460 (2013).
65. Krtolica, A., Parrinello, S., Lockett, S., Desprez, P.-Y. & Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: A link between cancer and aging. *Proc. Natl. Acad. Sci.* **98,** 12072–12077 (2001).
66. Kang, T. W. *et al.* Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* **479,** 547–551 (2011).
67. Hoenicke, L. & Zender, L. Immune surveillance of senescent cells-biological significance in cancer-and non-cancer pathologies. *Carcinogenesis* **33,** 1123–1126 (2012).
68. Nelson, G. *et al.* A senescent cell bystander effect: Senescence-induced senescence. *Aging Cell* **11,** 345–349 (2012).
69. Hubackova, S., Krejcikova, K., Bartek, J. & Hodny, Z. IL1-and TGFβ-Nox4 signaling, oxidative stress and DNA damage response are shared features of replicative, oncogene-induced, and drug-induced paracrine 'Bystander senescence'. *Aging (Albany. NY).* **4,** 932–951 (2012).
70. Dörr, J. R. *et al.* Synthetic lethal metabolic targeting of cellular senescence in cancer therapy. *Nature* **501,** 421–425 (2013).
71. Lee, M. & Lee, J.-S. Exploiting tumor cell senescence in anticancer therapy. *BMB Rep.* **47,** 51–59 (2014).
72. Neves, J., Demaria, M., Campisi, J. & Jasper, H. Of flies, mice, and men: Evolutionarily conserved tissue damage responses and aging. *Dev. Cell* **32,** 9–18 (2015).
73. Sharpless, N. E. & Sherr, C. J. Forging a signature of in vivo senescence. *Nat. Rev. Cancer* **15,** 397–408 (2015).
74. Wolf, J., Rose-John, S. & Garbers, C. Interleukin-6 and its receptors: a highly regulated and dynamic system. *Cytokine* **70,** 11–20 (2014).
75. Ataie-Kachoie, P., Pourgholami, M. H., Richardson, D. R. & Morris, D. L. Gene of the month: Interleukin 6 (IL-6). *J. Clin. Pathol.* **67,** 932–937 (2014).
76. Kaushik, S., Singh, R. & Cuervo, A. M. Autophagic pathways and metabolic stress. *Diabetes, Obes. Metab.* **12,** 4–14 (2010).
77. Douglas, P. M., Summers, D. W. & Cyr, D. M. Molecular chaperones antagonize proteotoxicity by differentially modulating protein aggregation pathways. *Prion* **3,** 51–8 (2009).
78. Gidalevitz, T. Progressive Disruption of Cellular Protein Folding in Models of Polyglutamine Diseases. *Science (80-.).* **311,** 1471–1474 (2006).
79. Douglas, P. M. & Dillin, A. Protein homeostasis and aging in neurodegeneration. *J. Cell Biol.* **190,** 719–729 (2010).
80. Chan, E. Y. mTORC1 Phosphorylates the ULK1-mAtg13-FIP200 Autophagy Regulatory Complex. *Sci. Signal.* **2,** pe51-pe51 (2009).
81. Inoki, K., Kim, J. & Guan, K.-L. AMPK and mTOR in Cellular Energy Homeostasis and Drug Targets. *Annu. Rev. Pharmacol. Toxicol* **52,** 381–400 (2012).
82. Capparelli, C. *et al.* CDK inhibitors (p16/p19/p21) induce senescence and autophagy in cancer-associated fibroblasts, 'fueling' tumor growth via paracrine interactions, without an increase in neo-angiogenesis. *Cell Cycle*

(2012). doi:10.4161/cc.21884
83. Dulic, V. in *Methods in Molecular Biology* **965,** 15–35 (Humana Press, Totowa, NJ, 2013).
84. Narita, M. *et al.* Spatial Coupling of mTOR and Autophagy Augments Secretory Phenotypes. *Science (80-.).* **332,** 966–970 (2011).
85. Nam, H. Y., Han, M. W., Chang, H. W., Kim, S. Y. & Kim, S. W. Prolonged autophagy by MTOR inhibitor leads radioresistant cancer cells into senescence. *Autophagy* **9,** 1631–1632 (2013).
86. Herranz, N. *et al.* mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. *Nat. Cell Biol.* **17,** 1205–1217 (2015).
87. Reitz, C. & Mayeux, R. Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers. *Biochem. Pharmacol.* **88,** 640–651 (2014).
88. Skaper, S. D. *Alzheimer's Disease and Amyloid: Culprit or Coincidence? International Review of Neurobiology* **102,** (Elsevier Inc., 2012).
89. Reinhard, C., Hébert, S. S. & De Strooper, B. The amyloid-β precursor protein: integrating structure with biological function. *EMBO J.* **24,** 3996–4006 (2005).
90. Zheng, H. & Koo, E. H. Molecular Neurodegeneration The amyloid precursor protein: beyond amyloid. *Mol. Neurodegener.* **1,** 1–12 (2006).
91. Hoareau, C. *et al.* Amyloid precursor protein cytoplasmic domain antagonizes reelin neurite outgrowth inhibition of hippocampal neurons. *Neurobiol. Aging* **29,** 542–553 (2008).
92. Lee, J. H. *et al.* Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. *Cell* **141,** 1146–1158 (2010).
93. Haung Yu, W. *et al.* Macroautophagy - A novel β-amyloid peptide-generating pathway activated in Alzheimer's disease. *J. Cell Biol.* **171,** 87–98 (2005).
94. Nixon, R. A. *et al.* Extensive Involvement of Autophagy in Alzheimer Disease: An Immuno-Electron Microscopy Study. *J. Neuropathol. Exp. Neurol.* **64,** 113–122 (2005).
95. Pluquet, O., Pourtier, A. & Abbadie, C. The unfolded protein response and cellular senescence. A Review in the Theme: Cellular Mechanisms of Endoplasmic Reticulum Stress Signaling in Health and Disease. *Am. J. Physiol. - Cell Physiol.* **308,** C415–C425 (2015).
96. Selkoe, D. J. Deciphering the genesis and fate of amyloid β-protein yields novel therapies for Alzheimer disease. *Journal of Clinical Investigation* **110,** 1375–1381 (2002).
97. Selkoe, D. J. Cell Biology of the Amyloid beta-Protein Precursor and the Mechanism of Alzheimer's Disease. *Annu. Rev. Cell Biol.* **10,** 373–403 (1994).
98. Cole, S. L. & Vassar, R. The Alzheimer's disease Beta-secretase enzyme, BACE1. *Mol. Neurodegener.* **2,** 22 (2007).
99. Vetrivel, K. S., Zhang, Y., Xu, H. & Thinakaran, G. Pathological and physiological functions of presenilins. *Mol. Neurodegener.* **1,** 4 (2006).
100. Allinson, T. M. J., Parkin, E. T., Turner, A. J. & Hooper, N. M. ADAMs Family Members As Amyloid Precursor Protein α-Secretases. *J. Neurosci. Res.* **74,** 342–352 (2003).
101. Müller, T., Meyer, H. E., Egensperger, R. & Marcus, K. The amyloid precursor protein intracellular domain (AICD) as modulator of gene expression, apoptosis, and cytoskeletal dynamics-Relevance for Alzheimer's disease. *Prog. Neurobiol.* **85,** 393–406 (2008).

102. Haapasalo, A. & Kovacs, D. M. The many substrates of presenilin/γ-secretase. *J. Alzheimer's Dis.* **25,** 3–28 (2011).
103. Li, Y. *et al.* Structural interactions between inhibitor and substrate docking sites give insight into mechanisms of human PS1 complexes. *Structure* **22,** 125–135 (2014).
104. Sato, T. *et al.* Active γ-secretase complexes contain only one of each component. *J. Biol. Chem.* **282,** 33985–33993 (2007).
105. Francis, R. *et al.* aph-1 and pen-2 are required for Notch pathway signaling, γ-secretase cleavage of βAPP, and presenilin protein accumulation. *Dev. Cell* **3,** 85–97 (2002).
106. Goutte, C., Tsunozaki, M., Hale, V. a & Priess, J. R. APH-1 is a multipass membrane protein essential for the Notch signaling pathway in Caenorhabditis elegans embryos. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 775–9 (2002).
107. Yu, G. *et al.* Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and βAPP processing. *Nature* **407,** 48–54 (2000).
108. Ahn, K. *et al.* Activation and intrinsic -secretase activity of presenilin 1. *Proc. Natl. Acad. Sci.* **107,** 21435–21440 (2010).
109. Esler, W. P. *et al.* Transition-state analogue inhibitors of γ-secretase bind directly to presenilin-1. *Nat. Cell Biol.* **2,** 428–434 (2000).
110. Li, Y. M. *et al.* Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6138–43 (2000).
111. Citron, M. *et al.* Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid β-protein in both transfected cells and transgenic mice. *Nat. Med.* **3,** 67–72 (1997).
112. Scheuner, D. *et al.* Secreted amyloid β-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* **2,** 864–870 (1996).
113. Chávez-Gutiérrez, L. *et al.* The mechanism of γ-Secretase dysfunction in familial Alzheimer disease. *EMBO J.* **31,** 2261–2274 (2012).
114. Shen, J. & Kelleher, R. J. The presenilin hypothesis of Alzheimer's disease: Evidence for a loss-of-function pathogenic mechanism. *Proc. Natl. Acad. Sci.* **104,** 403–409 (2007).
115. Xia, D. *et al.* Presenilin-1 knockin mice reveal loss-of-function mechanism for familial alzheimer's disease. *Neuron* **85,** 967–981 (2015).
116. Beher, D. *et al.* In vitro characterization of the presenilin-dependent γ-secretase complex using a novel affinity ligand. *Biochemistry* **42,** 8133–8142 (2003).
117. Gu, Y. *et al.* The presenilin proteins are components of multiple membrane-bound complexes that have different biological activities. *J. Biol. Chem.* **279,** 31329–31336 (2004).
118. Lai, M. T. *et al.* Presenilin-1 and presenilin-2 exhibit distinct yet overlapping γ-secretase activities. *J. Biol. Chem.* **278,** 22475–22481 (2003).
119. Edbauer, D. *et al.* Reconstitution of γ-secretase activity. *Nat. Cell Biol.* **5,** 486–488 (2003).
120. Li, T. *et al.* Increased expression of PS1 is sufficient to elevate the level and activity of γ-secretase in vivo. *PLoS One* **6,** (2011).
121. Carroll, C. M. & Li, Y. M. Physiological and pathological roles of the γ-secretase complex. *Brain Res. Bull.* **126,** 199–206 (2016).
122. Higaki, J., Quon, D., Zhong, Z. & Cordell, B. Inhibition of β-amyloid formation

identifies proteolytic precursors and subcellular site of catabolism. *Neuron* **14,** 651–659 (1995).
123. Klafki, H. W., Abramowski, D., Swoboda, R., Paganetti, P. A. & Staufenbiel, M. The carboxyl termini of β-amyloid peptides 1-40 and 1-42 are generated by distinct γ-secretase activities. *J. Biol. Chem.* **271,** 28655–28659 (1996).
124. Klafkia, H., Paganettib, P. A., Sommera, B. & Staufenbiela, M. Calpain inhibitor I decreases PA4 secretion from human embryonal kidney cells expressing P-amyloid precursor protein carrying the APP670 / 67 1 double mutation. *Neurosci. Lett.* **201,** 29–32 (1995).
125. Dovey, H. F. *et al.* Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J. Neurochem.* **76,** 173–181 (2001).
126. Lanz, T. A. The gamma-Secretase Inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl Ester Reduces Abeta Levels in Vivo in Plasma and Cerebrospinal Fluid in Young (Plaque-Free) and Aged (Plaque-Bearing) Tg2576 Mice. *J. Pharmacol. Exp. Ther.* **305,** 864–871 (2003).
127. Fleisher, A. S. *et al.* Phase 2 Safety Trial Targeting Amyloid β Production With a γ-Secretase Inhibitor in Alzheimer Disease. *Arch. Neurol.* **65,** 1031–1038 (2008).
128. Siemers, E. *et al.* Safety, tolerability, and changes in amyloid beta concentrations after administration of a gamma-secretase inhibitor in volunteers. *Clin. Neuropharmacol.* **28,** 126–132 (2005).
129. Siemers, E. R. *et al.* Safety, tolerability, and effects on plasma and cerebrospinal fluid amyloid-beta after inhibition of gamma-secretase. *Clin. Neuropharmacol.* **30,** 317–25 (2007).
130. Mitani, Y. *et al.* Differential Effects between -Secretase Inhibitors and Modulators on Cognitive Function in Amyloid Precursor Protein-Transgenic and Nontransgenic Mice. *J. Neurosci.* **32,** 2037–2050 (2012).
131. Campisi, J., Andersen, J. K., Kapahi, P. & Melov, S. Cellular senescence: A link between cancer and age-related degenerative disease? *Semin. Cancer Biol.* **21,** 354–359 (2011).
132. Ovadya, Y. & Krizhanovsky, V. Senescent cells: SASPected drivers of age-related pathologies. *Biogerontology* **15,** 627–642 (2014).
133. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* **153,** 1194–217 (2013).
134. Šoškić, V., Groebe, K. & Schrattenholz, A. Nonenzymatic posttranslational protein modifications in ageing. *Exp. Gerontol.* **43,** 247–257 (2008).
135. Hunter, S., Arendt, T. & Brayne, C. The senescence hypothesis of disease progression in alzheimer disease: An Integrated matrix of disease pathways for FAD and SAD. *Mol. Neurobiol.* **48,** 556–570 (2013).
136. Lleó, A. *et al.* Notch1 Competes with the Amyloid Precursor Protein for γ-Secretase and Down-regulates Presenilin-1 Gene Expression. *J. Biol. Chem.* **278,** 47370–47375 (2003).
137. Kinoshita, A., Shah, T., Tangredi, M. M., Strickland, D. K. & Hyman, B. T. The Intracellular Domain of the Low Density Lipoprotein Receptor-related Protein Modulates Transactivation Mediated by Amyloid Precursor Protein and Fe65. *J. Biol. Chem.* **278,** 41182–41188 (2003).
138. Lammich, S. *et al.* Presenilin-dependent intramembrane proteolysis of CD44 leads to the liberation of its intracellular domain and the secretion of an Aβ-like peptide. *J. Biol. Chem.* **277,** 44754–44759 (2002).
139. De Jonghe, C. Pathogenic APP mutations near the gamma-secretase

cleavage site differentially affect Abeta secretion and APP C-terminal fragment stability. *Hum. Mol. Genet.* **10,** 1665–1671 (2001).
140. Van Helmond, Z., Miners, J. S., Kehoe, P. G. & Love, S. Oligomeric Aβ in Alzheimer's disease: Relationship to plaque and tangle pathology, apoe genotype and cerebral amyloid angiopathy. *Brain Pathol.* **20,** 468–480 (2010).
141. Furukawa, K. *et al.* Increased Activity-Regulating and Neuroprotective Efficacy of α-Secretase-Derived Secreted Amyloid Precursor Protein Conferred by a C-Terminal Heparin-Binding Domain. *J. Neurochem.* **67,** 1882–1896 (2002).
142. Lee, D. H. S. & Wang, H. Y. Differential physiologic responses of α7 nicotinic acetylcholine receptors to β-amyloid1-40 and β-amyloid1-42. *J. Neurobiol.* **55,** 25–30 (2003).
143. Zou, K. *et al.* Amyloid β-protein (Aβ)1-40 protects neurons from damage induced by Aβ1-42 in culture and in rat brain. *J. Neurochem.* **87,** 609–619 (2003).
144. Roemer, K. in *Advances in Experimental Medicine and Biology* **727,** 223–240 (Springer US, 2012).
145. Fassa, A., Mehta, P. & Efthimiopoulos, S. Notch 1 interacts with the amyloid precursor protein in a numb-independent manner. *J. Neurosci. Res.* **82,** 214–224 (2005).
146. Kyriazis, G. A. *et al.* Numb endocytic adapter proteins regulate the transport and processing of the amyloid precursor protein in an isoform-dependent manner: Implications for Alzheimer disease pathogenesis. *J. Biol. Chem.* **283,** 25492–25502 (2008).
147. Hartmann, D., Tournoy, J., Saftig, P., Annaert, W. & De Stropper, B. Implication of APP Secretases in Notch Signaling. *J. Mol. Neurosci.* **17,** 171–181 (2001).
148. Woo, H.-N., Park, J.-S., Gwon, A.-R., Arumugam, T. V. & Jo, D.-G. Alzheimer's disease and Notch signaling. *Biochem. Biophys. Res. Commun.* **390,** 1093–1097 (2009).
149. Walton, M. *et al.* Do c-Jun, c-Fos, and amyloid precursor protein play a role in neuronal death or survival? *J. Neurosci. Res.* **53,** 330–342 (1998).
150. Ray, P. D., Huang, B. W. & Tsuji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* **24,** 981–990 (2012).
151. Caricasole, A. *et al.* Two sides of the same coin: Wnt signaling in neurodegeneration and neuro-oncology. *Biosci. Rep.* **25,** 309–327 (2005).
152. Souter, S. & Lee, G. Tubulin-independent tau in Alzheimer's disease and cancer: implications for disease pathogenesis and treatment. *Curr. Alzheimer Res.* **7,** 697–707 (2010).
153. Driver, J. A. & Lu, K. P. Pin1: A new genetic link between alzheimer's disease, cancer and aging. *Curr. Aging Sci.* **3,** 158–165 (2010).
154. Arendt, T. Alzheimer's disease as a disorder of dynamic brain self-organization. *Prog. Brain Res.* **147,** 355–378 (2004).
155. Arendt, T. Alzheimer's disease as a loss of differentiation control in a subset of neurons that retain immature features in the adult brain. *Neurobiol. Aging* **21,** 783–796 (2000).
156. Arendt, T. Synaptic plasticity and cell cycle activation in neurons are alternative effector pathways: The 'Dr. Jekyll and Mr. Hyde concept' of Alzheimer's disease or the yin and yang of neuroplasticity. *Prog. Neurobiol.* **71,** 83–248 (2003).

157. Herrup, K. Reimagining Alzheimer's Disease--An Age-Based Hypothesis. *J. Neurosci.* **30,** 16755–16762 (2010).
158. Frölich, L. The cholinergic pathology in Alzheimer's disease - Discrepancies between clinical experience and pathophysiological findings. *J. Neural Transm.* **109,** 1003–1014 (2002).
159. Tanaka, K. & Matsuda, N. Proteostasis and neurodegeneration: The roles of proteasomal degradation and autophagy. *Biochim. Biophys. Acta - Mol. Cell Res.* **1843,** 197–204 (2014).
160. Jing, H. *et al.* Opposing roles of NF-κB in anti-cancer treatment outcome unveiled by cross-species investigations. *Genes Dev.* **25,** 2137–2146 (2011).
161. Marcotte, R., Lacelle, C. & Wang, E. Senescent fibroblasts resist apoptosis by downregulating caspase-3. *Mech. Ageing Dev.* **125,** 777–783 (2004).
162. Hampel, B. *et al.* Apoptosis resistance of senescent human fibroblasts is correlated with the absence of nuclear IGFBP-3. *Aging Cell* **4,** 325–330 (2005).
163. Bassaneze, V., Miyakawa, A. A. & Krieger, J. E. A quantitative chemiluminescent method for studying replicative and stress-induced premature senescence in cell cultures. *Anal. Biochem.* **372,** 198–203 (2008).
164. Noda, T. & Ohsumi, Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* **273,** 3963–3966 (1998).
165. Krappmann, D., Wulczyn, F. G. & Scheidereit1, C. Different mechanisms control signal-induced degradation and basal turnover of the NF-icB inhibitor IcBa in vivo. *EMBO J.* **15,** 6716–6726 (1996).
166. Ki, T. *et al.* activity endotoxin : mOle ..: ar ships of struetui to and fction ; L0M. *FASEB J.* **8,** 217–225 (1994).
167. Sandor, F. & Buc, M. Toll-like receptors. II. Distribution and pathways involved in TLR signalling. *Folia Biologica* **51,** 188–197 (2005).
168. Nishimura, M. & Naito, S. Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. *Biol. Pharm. Bull.* **28,** 886–892 (2005).
169. Tan, F. C. C., Hutchison, E. R., Eitan, E. & Mattson, M. P. Are there roles for brain cell senescence in aging and neurodegenerative disorders? *Biogerontology* **15,** 643–660 (2014).
170. Radde, R. *et al.* Aβ42-driven cerebral amyloidosis in transgenic mice reveals early and robust pathology. *EMBO Rep.* **7,** 940–946 (2006).
171. Åslund, A. *et al.* Novel pentameric thiophene derivatives for in vitro and in vivo optical imaging of a plethora of protein aggregates in cerebral amyloidoses. *ACS Chem. Biol.* **4,** 673–684 (2009).
172. Reimann, M. *et al.* Tumor Stroma-Derived TGF-β Limits Myc-Driven Lymphomagenesis via Suv39h1-Dependent Senescence. *Cancer Cell* **17,** 262–272 (2010).
173. Schmitt, C. A., McCurrach, M. E., De Stanchina, E., Wallace-Brodeur, R. R. & Lowe, S. W. INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev.* **13,** 2670–2677 (1999).
174. Mullan, M. *et al.* A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N–terminus of β–amyloid. *Nat. Genet.* **1,** 345–347 (1992).
175. Yang, D. S. *et al.* Reversal of autophagy dysfunction in the TgCRND8 mouse model of Alzheimer's disease ameliorates amyloid pathologies and memory deficits. *Brain* **134,** 258–277 (2011).
176. Hardy, J. & Allsop, D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol. Sci.* **12,** 383–388 (1991).

177. Citron, M., Teplow, D. B. & Selkoe, D. J. Generation of amyloid β protein from its precursor is sequence specific. *Neuron* **14,** 661–670 (1995).
178. Walsh, D. M. *et al.* The APP family of proteins: similarities and differences. *Biochem. Soc. Trans.* **35,** 416–420 (2007).
179. Price, D. L. & Sisodia, S. S. Mutant genes in familial Alzheimer's disease and transgenic models. *Ann. Rev. Neurosci.* **21,** 479–505 (1998).
180. Wilson, C. A., Doms, R. W. & Lee, V. M.-Y. Intracellular APP Processing and Aβ Production in Alzheimer Disease. *J. Neuropathol. Exp. Neurol.* **58,** 787–794 (1999).
181. Price, D. L., Tanzi, R. E., Borchelt, D. R. & Sisodia, S. S. ALZHEIMER'S DISEASE: Genetic Studies and Transgenic Models. *Annu. Rev. Genet* **32,** 461–93 (1998).
182. Jämsä, A., Belda, O., Edlund, M. & Lindström, E. BACE-1 inhibition prevents the γ-secretase inhibitor evoked Aβ rise in human neuroblastoma SH-SY5Y cells. *J. Biomed. Sci.* **18,** 76 (2011).
183. Li, J., Uversky, V. N. & Fink, A. L. Effect of familial Parkinson's disease point mutations A30P and A53T on the structural properties, aggregation, and fibrillation of human α-synuclein. *Biochemistry* **40,** 11604–11613 (2001).
184. Price, D. L. *et al.* The value of transgenic models for the study of neurodegenerative diseases. *Ann. N. Y. Acad. Sci.* **920,** 179–91 (2000).
185. Karch, C. M., Cruchaga, C. & Goate, A. M. Alzheimer's disease genetics: From the bench to the clinic. *Neuron* **83,** 11–26 (2014).
186. Ewald, J. A., Desotelle, J. A., Wilding, G. & Jarrard, D. F. Therapy-induced senescence in cancer. *Journal of the National Cancer Institute* **102,** 1536–1546 (2010).
187. Sheng, J. G. *et al.* Lipopolysaccharide-induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid β peptide in APPswe transgenic mice. *Neurobiol. Dis.* **14,** 133–145 (2003).
188. Malavolta, M. *et al.* Peripheral mononuclear cell rejuvenation for senescence surveillance in Alzheimer disease. *Curr. Pharm. Des.* **19,** 1720–6 (2013).
189. McCray, B. A. & Taylor, J. P. The role of autophagy in age-related neurodegeneration. *NeuroSignals* **16,** 75–84 (2007).
190. Nixon, R. A., Yang, D. S. & Lee, J. H. Neurodegenerative lysosomal disorders: A continuum from development to late age. *Autophagy* **4,** 590–599 (2008).
191. Bellettato, C. M. & Scarpa, M. Pathophysiology of neuropathic lysosomal storage disorders. *J. Inherit. Metab. Dis.* **33,** 347–362 (2010).
192. Cherra, S. J., Dagda, R. K. & Chu, C. T. Review: Autophagy and neurodegeneration: Survival at a cost? *Neuropathol. Appl. Neurobiol.* **36,** 125–132 (2010).
193. Kroemer, G. & Jäättelä, M. Lysosomes and autophagy in cell death control. *Nat. Rev. Cancer* **5,** 886–897 (2005).
194. Ihara, Y., Morishima-Kawashima, M. & Nixon, R. The ubiquitin-proteasome system and the autophagic-lysosomal system in Alzheimer disease. *Cold Spring Harb. Perspect. Med.* **2,** (2012).
195. Yang, D.-S. *et al.* Neuronal Apoptosis and Autophagy Cross Talk in Aging PS/APP Mice, a Model of Alzheimer's Disease. *Am. J. Pathol.* **173,** 665–681 (2008).
196. Heinrich, M. *et al.* Cathepsin D targeted by acid sphingomyelinase- derived ceramide. *EMBO J.* **18,** 5252–5263 (1999).
197. Bahr, B. A. & Bendiske, J. The neuropathogenic contributions of lysosomal

dysfunction. *J. Neurochem.* **83,** 481–489 (2002).
198. Florez-McClure, M., Hohsfield, L., Fonte, G., Bealor, M. & Link, C. D. Decreased Insulin-Receptor Signalling Promotes the Autophagic Degradation of b-Amyloid Peptide in C. elegans. *Autophagy* **3,** 569–580 (2007).
199. Takahashi, R. H. *et al.* Intraneuronal Alzheimer Aβ42 accumulates in multivesicular bodies and is associated with synaptic pathology. *Am. J. Pathol.* **161,** 1869–1879 (2002).
200. Neely, K. M., Green, K. N. & LaFerla, F. M. Presenilin Is Necessary for Efficient Proteolysis through the Autophagy-Lysosome System in a -Secretase-Independent Manner. *J. Neurosci.* **31,** 2781–2791 (2011).
201. Haass, C. & Selkoe, D. J. A technical KO of amyloid-β peptide. *Nature* **391,** 339–340 (1998).
202. Ferreiro, E., Resende, R., Costa, R., Oliveira, C. R. & Pereira, C. M. F. An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity. *Neurobiol. Dis.* **23,** 669–678 (2006).
203. Ferreiro, E., Oliveira, C. R. & Pereira, C. M. F. Involvement of endoplasmic reticulum Ca2+ release through ryanodine and inositol 1,4,5-triphosphate receptors in the neurotoxic effects induced by the amyloid-β peptide. *J. Neurosci. Res.* **76,** 872–880 (2004).
204. Nishitsuji, K. *et al.* The E693Delta mutation in amyloid precursor protein increases intracellular accumulation of amyloid beta oligomers and causes endoplasmic reticulum stress-induced apoptosis in cultured cells. *Am. J. Pathol.* **174,** 957–69 (2009).
205. Resende, R., Ferreiro, E., Pereira, C. & Oliveira, C. R. ER stress is involved in Aβ-induced GSK-3β activation and tau phosphorylation. *J. Neurosci. Res.* **86,** 2091–2099 (2008).
206. Resende, R., Ferreiro, E., Pereira, C. & Resende de Oliveira, C. Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: Involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. *Neuroscience* **155,** 725–737 (2008).
207. Schlachetzki, J. C. M., Saliba, S. W. & de Oliveira, A. C. P. Studying neurodegenerative diseases in culture models. *Rev. Bras. Psiquiatr.* **35,** (2013).
208. Karran, E. & Hardy, J. A critique of the drug discovery and phase 3 clinical programs targeting the amyloid hypothesis for Alzheimer disease. *Ann. Neurol.* **76,** 185–205 (2014).
209. De Strooper, B. Lessons from a failed γ-secretase Alzheimer trial. *Cell* **159,** 721–726 (2014).
210. Lauritzen, I. *et al.* The -Secretase-Derived C-Terminal Fragment of APP, C99, But Not A , Is a Key Contributor to Early Intraneuronal Lesions in Triple-Transgenic Mouse Hippocampus. *J. Neurosci.* **32,** 16243–16255 (2012).
211. Lauritzen, I. *et al.* Intraneuronal aggregation of the β-CTF fragment of APP (C99) induces Aβ-independent lysosomal-autophagic pathology. *Acta Neuropathol.* **132,** 1–20 (2016).
212. Selkoe, D. *et al.* Beta-amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and nonneural tissues. *Proc. Natl. Acad. Sci.* **85,** 7341–7345 (1988).
213. Joachim, C. L., Mori, H. & Selkoe, D. J. Amyloid β-protein deposition in tissues other than brain in Alzheimer's disease. *Nature* **341,** 226–230 (1989).
214. Yamada, T., Sasaki, H., Dohura, K., Goto, I. & Sakaki, Y. Structure and

expression of the alternatively-spliced forms of mRNA for the mouse homolog of Alzheimer's disease amyloid beta protein precursor. *Biochem. Biophys. Res. Commun.* **158,** 906–912 (1989).
215. Akaaboune, M. *et al.* Developmental Regulation of Amyloid Precursor Protein at the Neuromuscular Junction in Mouse Skeletal Muscle. *Mol. Cell. Neurosci.* **15,** 355–367 (2000).
216. HERZOG, V. Biological roles of APP in the epidermis. *Eur. J. Cell Biol.* **83,** 613–624 (2004).
217. GALLOWAY, S., JIAN, L., JOHNSEN, R., CHEW, S. & MAMO, J. β-Amyloid or its precursor protein is found in epithelial cells of the small intestine and is stimulated by high-fat feeding. *J. Nutr. Biochem.* **18,** 279–284 (2007).
218. Lee, Y.-H. *et al.* Amyloid Precursor Protein Expression Is Upregulated in Adipocytes in Obesity. *Obesity* **16,** 1493–1500 (2008).
219. Sandbrink, R., Masters, C. L. & Beyreuther, K. βA4-Amyloid protein precursor mRNA isoforms without exon 15 are ubiquitously expressed in rat tissues including brain, but not in neurons. *J. Biol. Chem.* **269,** 1510–1517 (1994).
220. Puig, K. L. & Combs, C. K. Expression and function of APP and its metabolites outside the central nervous system. *Exp. Gerontol.* **48,** 608–611 (2013).
221. Baker, D. J. *et al.* Clearance of p16 Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* **479,** 232–236 (2011).
222. Muñoz-Espín, D. & Serrano, M. Cellular senescence: From physiology to pathology. *Nat. Rev. Mol. Cell Biol.* **15,** 482–496 (2014).
223. Vijg, J. & Campisi, J. Puzzles, promises and a cure for ageing. *Nature* **454,** 1065–1071 (2008).
224. Agholme, L., Hallbeck, M., Benedikz, E., Marcusson, J. & Kgedal, K. Amyloid-β secretion, generation, and lysosomal sequestration in response to proteasome inhibition: Involvement of autophagy. *J. Alzheimer's Dis.* **31,** 343–358 (2012).
225. Cheng, N., Jiao, S., Gumaste, A., Bai, L. & Belluscio, L. APP Overexpression Causes Aβ-Independent Neuronal Death through Intrinsic Apoptosis Pathway. *eNeuro* **3,** 1–12 (2016).
226. Li, J. Q., Yu, J. T., Jiang, T. & Tan, L. Endoplasmic reticulum dysfunction in Alzheimer's disease. *Mol. Neurobiol.* **51,** 383–395 (2015).
227. Wolfe, M. S. Inhibition and Modulation of γ-Secretase for Alzheimer's Disease. *Neurotherapeutics* **5,** 391–398 (2008).

ACKNOWLEDGEMENT

I would like to thank Prof. Clemens Schmitt, for providing me the opportunity to pursue my PhD in his lab. I am extremely grateful for the years of mentorship and thoughtful discussions that encouraged me to become a better scientist. I would also like to thank Dr. Soyoung Lee for her continuous support and guidance throughout the project. She has provided me with lots of encouragement and insightful ideas whenever I was stuck in a challenging situation.

I'm grateful to many of my senior colleagues. Dr. Yong Yu has been a very calm and helpful senior colleague. He never lost his patience while answering my many questions about everyday lab work and other PhD life related problems. Dr. Kolja Schleich has provided me with data analysis of the microarray data along with heat maps and GSEA plots. He was always helpful and readily provided me with updated versions of graphs using his beloved "R". Dr. Animesh Bhattacharya was extremely generous with his comments and suggestions on my presentations and my work. I'm especially grateful to Dr. Fatimunnisa Qadri. She was very kind for offering help, even though she was from another lab in MDC. She helped me with some mouse experiments, as she is a neurobiology expert.

I enjoyed working with and learning from my other fellow PhD students and colleagues. Lianjie Li and Bin Yue taught me helpful tips and tricks to make life in lab easier. Moreover, we had interesting discussions about the relations between India and China, while trying to maintain ours. I am thankful to Stefanie Brückner for her encouragement with my thesis and Harikrishnan Radhakrishnan for his time, since he was extremely helpful while I was writing my thesis.

Our lab technicians were my saviors, especially Marika Drescher, Anja Wolf and Andrea Herrmann. They assisted me with my experiments whenever I was over loaded with work. They were very accessible whenever I was handicapped with my incompetence in the German language. I am also very grateful to Dr. Eleanor Eife-Horn, Wiebke Höpner and Birgit Henkel for their constant help with official and sometimes non-official matters. They are some of the nicest people I have ever come across.

I am forever indebted to my parents for always supporting me and encouraging me with all my decisions. They have been my pillars of support through all my

adversities in this country, even though they were thousands of miles away. I feel very lucky to have such strong emotional support from them. I also feel fortuitous to have my grandparents' blessings. Especially my grandmother who tried to act as a therapist and always sent me "positive vibes".

Most importantly and most deservingly, I dedicate this thesis to my husband, Sanandan Malhotra, who has been my rock. I could not have done any of this without him. He has supported me, encouraged me, been patient with me through thick and thin. I would not have been able to get through any of this without him by my side (figuratively).

Finally, Berlin became a very big part of my life and I will cherish every moment in this city. I made many friends here and some of them I hope to keep for a lifetime. I am indebted to BSIO for making all of this possible.

STATEMENT

I hereby declare that I completed the doctoral thesis independently based on the stated resources and aids. I have not applied for a doctoral degree elsewhere and do not have a corresponding doctoral degree. I have not submitted the doctoral thesis, or parts of it, to another academic institution and the thesis has not been accepted or rejected. I declare that I have acknowledged the Doctoral Degree Regulations which underlie the procedure of the Faculty of Life Sciences of Humboldt-Universität zu Berlin, as amended on 5th March 2015. Furthermore, I declare that no collaboration with commercial doctoral degree supervisors took place, and that the principles of Humboldt-Universität zu Berlin for ensuring good academic practice were abided by.

Date / signature